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(54) Title: MULTIDRUG RESISTANCE ASSOCIATED PROTEINS AND USES THEREOF

(57) Abstract

The present invention relates to multidrug resistance, specifically to multidrug resistant protein 4(MRP4) and uses thereof. The present invention provides nucleic acid encoding MRP4, MRP4 protein, antibody reactive to MRP4 and discloses MRP4 as perhaps the first mammalian efflux pump described for nucleoside analogs. The present invention provides the first example of a role of MRP4 in drug resistance. Certain patients who develop drug resistance to anti–microbial therapy or anti–cancer therapy may develop cellular resistance mediated by MRP4. Accordingly, the present invention possesses both diagnostic and therapeutic utility as diagnostic kits, including drug assays and screens are contemplated, as well as pharmaceutical compositions and the corresponding methods of their respective use. For example, a diagnostic kit is disclosed that may be used in order to facilitate determination of patient susceptibility to MRP4 mediated drug resistance.

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MULTIDRUG RESISTANCE ASSOCIATED PROTEINS AND USES THEREOF

GOVERNMENTAL SUPPORT

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Throughout this application, certain publications are referenced by number. Full citations for these publications may be found listed at the end of the specification and preceding the Claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art. A Sequence Listing is provided.

FIELD OF THE INVENTION

The present invention relates generally to to multi-drug resistance, and more particularly to materials such as multi-drug resistant proteins (MRP), and to possible diagnostic and therapeutic uses thereof. The invention further relates to methods for identifying treatments refractive to drug resistance.

BACKGROUND OF THE INVENTION

Microbial and cellular resistance to drug therapy is a major and long-standing problem to the treatment of disease and infection, including cancer. Cross-resistance between different anti-microbial and anti-cancer agents, which are structurally and functionally distinct, is a relatively common phenomenon called multi-drug resistance (MDR).

With respect to cancers, some malignant tumors respond poorly to chemotherapy,

indicating that the target cells are intrinsically resistant. Other tumors initially respond well to chemotherapy, but appear to develop resistance, indicating a selection process or cellular response to the chemotherapeutic agent(s). The broad-spectrum resistance characteristic of MDR, therefore, is of great clinical significance.

MDR was initially described in cultured tumor cells which following selection for resistance to a single anti-tumor agent became resistant to a range of chemically diverse anti-cancer agents (52). These MDR cells exhibited a decrease in intracellular drug accumulation due to active efflux by transporter proteins. The so-called "multi-drug transporters" are membrane proteins capable of expelling a broad range of toxic molecules from the cell (53). These multi-drug transporters belong to the ATP-binding cassette (ABC) superfamily of transport proteins that utilize the energy of ATP hydrolysis for activity (53, 57). In microorganisms, multi-drug transporters play an important role in conferring antibiotic resistance on pathogens.

Several mechanisms have been described as responsible for MDR. The most well characterized gene conferring drug resistance by an ATP-dependent efflux mechanism is the MDR1 gene product, P-glycoprotein (Pgp), a member of the ABC cassette family of transporters. Pgp removes hydrophobic drugs of diverse chemical structures from cells as an efflux pump (55).

Another transporter protein, capable of conferring drug resistance, the multi-drug resistance protein (MRP), has been identified in a number of MDR human tumor cell lines that do not appear to express Pgp (52). The presence of MRP at the cell surface of such cells has been associated with alterations in drug accumulation and distribution (52). Expression of MRP causes a form of multi-drug resistance similar to that conferred by Pgp (52). The two proteins, however, are only distantly related. MRP has also been shown to be a primary active transporter of a structurally diverse range of organic anionic conjugates. Like Pgp, MRP has a

broad substrate specificity. In addition to hydrophobic compounds, MRP is able to transport metallic oxyanions and glutathione and other conjugates, including peptidyl leukotrienes (52). This is in contrast to Pgp. (Stride, BD, et al., 1997, Mol. Pharmacol., 52:344-53). The mechanism by which MRP transports these compounds and mediates multi-drug resistance is not understood. In addition, topoisomerase II has been associated with MDR. Like Pgp, MRP is expressed in normal human tissues in addition to tumor cells (52). In normal cells, MRP appears to be located within the cytoplasm, indicating that it may function differently in normal cells as compared with tumor cells (52). Homologs of human Pgp and MRP have been found in microorganisms such as Plasmodium falciparum, candida albicans, Saccharomyces cerevisiae and Lactococcus lactis (53).

Although MDR1 was cloned some time ago, proteins in animal cells that were functionally similar were not readily identified. MRP has been described which in some cell types confer a drug resistance phenotype similar to the MDR1 gene (58). The prototype MRP1 gene was first described in 1992. Subsequently, MRP2 (cMOAT) was cloned. Both MRP1 and MRP2 act to efflux anionic compounds, including drugs or endogenous compounds. Several yeast MRP homologues have been identified (49) and recently, additional human homologues have been identified in the EST databases. Particularly, Borst and colleagues searched the EST database and identified four additional family members (MRP2, MRP3, MRP4, and MRP5). Nonetheless, the human MRP homologues have until now remained functionally undefined. MRP3 has been described as exhibiting high expression in some cell lines but not in others, with overexpression of MRP3 in resistant lines being identified in several doxorubicin-resistant and cisplatin-resistant cell lines (49). MRP5 was identified as being very widely expressed. MRP4 in contrast to MRP3 and MRP5, was not reportedly overexpressed in any cell line analyzed (49). Importantly, the EST-based primary MRP4 sequence determined lacks several crucial pieces of information including: (1) a classic Walker A motif which is a signature of the ATP-binding domain found in ABC cassette transporter members; (2) more than 90% of the protein sequence; and (3) any functional marker. MRP3,

MRP4 and MRP5 have been localized to a different chromosome than MRP1 and MRP2, indicating that they are not merely alternative splicing products (49).

ABC transporters are integral membrane proteins involved in ATP-dependent transport across biological membranes. Members of this superfamily play roles in a number of phenomena of biomedical interest, including cystic fibrosis (CFTR) and multi-drug resistance. Many ABC transporters are predicted to consist of two functional domains, a membrane-spanning domain and a cytoplasmic domain. The latter contain conserved nucleotide-binding motifs with the former containing substrate binding or recognition sites. Attempts to determine the structure of ABC transporters and of their separate domains have not yet been successful (57).

The ABC transporters of glutathione S-conjugates and related amphiphilic anions have been identified as MRP1 and MRP2. These 190-kDa membrane glycoproteins have been cloned. MRP1 and MRP2 have been shown to be unidirectional, ATP-driven, export pumps with an amino acid identity of 49% in humans. MRP1 is detected in the plasma membrane of many cell types, including erythrocytes. MRP2, also known as canalicular MRP (cMRP) or canalicular multispecific organic anion transporter (cMOAT), has been localized to the apical domain of polarized epithelia, such as the hepatocyte canalicular membrane and kidney proximal tubule luminal membrane. Physiologically important substrates of both transporters include glutathione S-conjugates, such as leukotriene C4, as well as bilirubin glucuronides, 17 ß-glucuronosyl estradiol and glutathione disulfide. Both transporters have been associated with multiple drug resistance of malignant tumors because of their capacity to pump drug conjugates and drug complexes across the plasma membrane into the extracellular space. The substrate specificity of MRP1 and MRP2 studied in inside-out oriented membrane vesicles is very different from MDR1 (Pgp). MRP1 and MRP2 have been called conjugate transporting ATPases, functioning in detoxification and, because of their role in glutathione disulfide export, in the defense against oxidative stress (54).

A cDNA encoding another ATP-binding cassette transporter, MOAT-B, has been reportedly cloned and mapped (56). Comparison of the MOAT-B predicted protein with other transporters revealed that it is most closely related to MRP, cMOAT, and the yeast organic anion transporter YCF1. Although MOAT-B is closely related to these transporters, it is distinguished by the absence of a approximately 200 amino acid NH_2-terminal hydrophobic extension that is present in MRP and cMOAT and which is predicted to encode several transmembrane spanning segments. In addition, the MOAT-B tissue distribution is distinct from MRP and cMOAT. In contrast to MRP, which is widely expressed in tissues, including liver, and cMOAT, the expression of which is largely restricted to liver, the MOAT-B transcript is widely expressed, with particularly high levels in prostate, but is barely detectable in liver. (6).

The sequence and structural similarity between eukaryotic and prokaryotic ABC transporters is striking. The sequence similarity extends beyond the conserved components (the nucleotide-binding sequence motif, *i.e.* Walker motifs, and the ABC signature sequence) and includes several hundred amino acids on either side of the Walker motifs. Functionally, this suggests conservation between the coupling of ATP binding and ATP hydrolysis, with both processes necessary to facilitate substrate transport. The functional conservation is observed from prokaryotic to eukaryotes even though the substrates may be markedly different. Further evidence that the ABC transporters can be grouped together is found in an analysis of their predicted secondary structures which shows these molecules possess remarkably similar secondary structures across the entire phylogenetic spectrum of ABC transporters.

The recently completed sequence of the yeast genome revealed that over 29 proteins belong to the ABC transporter family (59). Despite the sequence and structural similarity a diverse array of substrates and functions are attributed to the ABC transporters. For example, in *S. cerevisiae*, the STE6 protein is necessary for the secretion of essential mating protein, α-factor; in *D. melanogaster*, the white and

brown gene products may transfer pigment proteins; in mammals, MRP1 transports some cancer therapeutic agents and glutathione conjugates; and CFTR serves as a ion channel. Thus, it is clear that simply being a member of the large ABC transport family does not in any way define the type of substrate transported nor does a predicted secondary structure. Further, many of the substrates transported are structurally diverse and do not share clearly definable molecular signatures.

Antiviral therapies used to treat HIV and other DNA virus infections include acyclic nucleoside phosphonates. These represent a new class of nucleotide analogs that exhibit potent and selective activity against a variety of both DNA and RNA viruses, including the human immunodeficiency virus and hepatitis B virus.

The acyclic nucleoside phosphonate PMEA (9-(2phosphonylmethoxyethyl)adenine) is a broad-spectrum agent that exhibits potent antiviral activity against various DNA viruses and retroviruses, including HIV (1-14). PMEA and its lipophilic prodrug bispom-PMEA (15) have entered phase I clinical trials as treatment for HIV infections (6,17). PMEA acts as a stable monophosphate analog of AMP and dAMP, and its antiviral activity is thought to require activation to the diphosphate derivative PMEApp, which then acts to inhibit viral DNA polymerases with relative sparing of cellular DNA replication (8, 11, 18). However, the exact mode of metabolism and action of PMEA and related acyclic phosphonate analogs remains unclear. Studies have suggested that PMEA enters cells via endocytosis and is further metabolized to PMEAp and PMEApp by cellular enzymes (19). One report suggested that the anabolism of PMEA may involve direct conversion to PMEApp via PRPP synthetase, although direct evidence for this mechanism in intact cells has not been obtained (11).

Sequential intracellular phosphorylation of the nucleoside and phosphonate analogs is essential for the bioactivation of these compounds (6-8). The phosphorylated metabolites function as anti-HIV drugs by inhibiting the reverse transcriptase (RT) enzyme of HIV. It has been well documented that achieving optimal intracellular

concentrations of the phosphorylated biotransformation products is important for exerting the anti-viral effect (9, 10).

For a clinically efficacious treatment of HIV infection, long-term use of the nucleoside RT inhibitors is a very common practice. The development of drug resistance to these compounds is well documented in the literature. Several reasons for the development of drug resistance have been observed: (i) These nucleoside analogs and the acyclic nucleoside phosphonates select for various mutations in the RT that confers varying degrees of viral resistance to the different analogs (11, 12). (ii) A decrease in the enzymes involved in the biotransformation of the drug to the active metabolite leads to cellular resistance (13). (iii) A change in the transport of the drug, either decreased uptake or increased flux such that optimal intracellular levels are not attained could lead to cellular resistance (48).

Because PMEA is a nucleotide analog which enters cells by a nonspecific process of endocytosis, decreased cellular influx is unlikely as a mode of cellular resistance. Nevertheless, studies have revealed that resistance to PMEA and similar compounds (*e.g.* AZT) appears secondary to decreased intracellular accumulation, thus limiting the therapeutic effectiveness of these compounds. Moreover, different T-cell lines do not achieve comparable intracellular concentrations of these compounds despite comparable extracellular concentrations.

These findings are consistent with the suggestion that intracellular accumulation may negatively impact pharmacologic antiviral therapeutic efficacy and imply enhanced transport of these compounds out of the cell as an important unrecognized mechanism in therapeutic failure. Accordingly, the identification of inhibitors and substrates associated with drug efflux and the development of strategies for controlling this activity, would therefore be expected to have substantial therapeutic impact, and it is toward the fulfillment of this and other like objectives that the present invention is directed.

SUMMARY OF THE INVENTION

In its broadest aspect, the present invention relates to a protein hereinafter referred to and exemplified by multi-drug resistance protein 4 (MRP4), and extends to nucleic acid molecules encoding it, cells that express it, and to its variants, including conserved variants, antagonists including antibodies, analogs, and mimics, including small molecules.

The invention further relates to finding respecting the role of MRP4 in drug efflux particularly in humans, and to the diagnostic and therapeutic applications of this activity, such as the ability to enhance and extend drug and like therapies where efficacy is dependent upon the development and maintenance of consistent, high intracellular levels of the therapeutic agent. In such event, the invention extends to, *e.g.* methods for administering anti-cancer or anti-HIV therapeutic agents, which methods include modulating the expression of MRP4 and/or administering antagonists or other agents that suppress the drug efflux activity of MRP4.

Further, the invention extends to the novel human T cell line, termed CEMr-1 has been generated which expresses a multi-drug-resistant phenotype to a variety of unrelated clinically active anti-HIV nucleoside agents such as zidovudine (AZT), lamivudine (3TC), and the acyclic phosphonate analogs such as PMEA and PMPA. (48). This multi-drug-resistant phenotype is associated with an ATP-dependent efflux of the mono phosphorylated congeners from these cells. These results suggested the involvement of an efflux pump similar to those described for many cancer drugs. However, biochemical characterization of the resistant cells revealed that the known P-glycoprotein pump was not responsible for drug efflux in CEMr-1 cells

As stated above, the present invention further extends to and provides a method for modulating drug resistance by controlling the presence, activity and/or the expression of MRP4, its mimics, antagonists, analogs, congeners, active fragments,

conserved variants, and mixtures. The present invention represents the first example of a role of MRP4 in drug resistance. Moreover and as stated above, these results suggest that MRP4 functions as an organic anion transporter that is capable of effluxing nucleoside analogs and other drugs with anionic functionality. Indeed this represents the first mammalian pump described for nucleoside analogs with anionic functionality (*i.e.* all analogs with a purine and pyrimidine selection). It is expected that certain patients who either develop resistance to therapy without displaying viral resistance may develop cellular resistance by this mechanism. Alternatively, variation between individual's MRP4 expression may determine therapeutic efficacy.

The present invention discloses that MRP4 functions as a drug efflux protein. MRP4 antibodies are also disclosed by the instant invention, and their diagnostic and therapeutic use is also contemplated.

MRP4 may represent the first gene described in mammals that effluxes intracellular nucleotides, thus representing an important molecule regulating nucleotide balance within cells, with therapeutic relevance.

It is another object of the present invention to provide an antibody capable of specifically binding to the provided protein without substantially cross-reacting with non MRP4 proteins or homologs thereof under conditions permissive to antibody binding.

Also an object of the present invention is to provide a diagnostic kit for identifying individuals resistant to anti-retroviral agent therapy comprising the provided probe or antibody.

It is also an object of the present invention to provide a kit for identifying a compound which is refractive to MRP4 efflux comprising the provided probe.

It is an object of the present invention to provide a nucleic acid probe capable of specifically hybridizing with the provided nucleic acid.

Additionally, it is an object of the present invention to provide a method of identifying the provided MRP4 protein in a sample.

It is a further object of the present invention to provide a method for identifying a nucleic acid in a sample which encodes MRP4 protein.

Still a further object of the present invention is to provide a method for identifying a compound that modulates expression of MRP4.

Further still, it is an object of the present invention to provide a method for identifying a compound capable of modulating MRP4 protein activity.

Also an object of the present invention is to provide a method of modulating MRP4 protein activity in a sample, comprising contacting the sample with the modulator compound identified by the provided method.

Yet another object of the present invention is to provide a pharmaceutical composition which comprises the identified modulator compound and a pharmaceutically acceptable carrier.

Still a further object of the present invention is to provide a method for treating a condition in a subject which comprises administering to the subject an amount of the provided pharmaceutical composition, effective to treat the condition in the subject.

A further still object of the present invention is to provide a method for identifying subjects at risk for resistance to anti-microbial agent therapy.

Yet another object of the present invention is to provide a method for identifying an

anti-microbial agent which is refractive to MRP4 efflux activity.

It is still further object of the present invention to provide a transgenic non-human animal whose somatic and germ cells contain and express a gene encoding MRP4 protein, the gene having been introduced into the animal or an ancestor of the animal at an embryonic stage and wherein the gene may be operably linked to an inducible promoter element.

Other objects and advantages will become apparent to those skilled in the art from a consideration of the ensuing description which proceeds with reference to the illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Cytostasis (inhibition of cell proliferation) of PMEA in CEM-SS and CEM-rl cells. Exponential cultures were incubated with PMEA, and the increases in cell numbers were monitored after 48-hr incubations, using a Coulter counter, as described herein. The results are the mean from two independent experiments done in duplicate.

FIGURE 2. Uptake and metabolism of [^{3}H]PMEA in CEM-SS and CEM-rl cells. Exponentially growing cultures were incubated with 10 μM [^{3}H]PMEA, and at various intervals the cell extracts were analyzed for PMEA and its metabolites by ion exchange HPLC, as described herein. These results are from an experiment that was repeated twice, with identical results.

FIGURES 3A-3C. Uptake of [^{3}H]bispom-PMEA in CEM-SS and CEM-rl cells. Exponentially growing cultures were incubated with 1 μM [^{3}H]bispomPMEA, and at the indicated times incubations were terminated by centrifugation of cells through an inert oil phase. The amounts of bispomPMEA internalized at various intervals of incubation are shown. The data are from a typical experiment, which has been

repeated several times with identical results.

FIGURES 4A-4B. Efflux of PMEA from [^{3}H]bispom-PMEA-treated cultures. Exponentially growing cultures of CEM-SS and CEM-rl cells were incubated for 15 minutes with 1 μM or 2 μM [^{3}H]bispom-PMEA, respectively. The cell extracts and media were analyzed by ion exchange HPLC as described herein.

FIGURE 5. Uptake of AZT in CEM-SS and CEM-r1 cells. cells were incubated with 10 μM or 100 μM [^{3}H]AZT for time periods ranging from 10-60 seconds Transport was terminated by the addition of cold PBS containing a transport inhibitor and centrifuging the cells through oil in a microfuge. The radioactivity was determined in a liquid scintillation counting.

FIGURES 6A-6B. AZTMP efflux. Cells were loaded with 10 μM of [^{3}H]AZT for 1 h at 27°C. Radioactivity was determined immediately after removal of radioactive medium and washing the cells with ice-cold PBS. At various time periods ranging from 2 to 20 minutes samples of the cells were taken and the level of radioactivity remaining in the cells as AZTMP and that excreted into the medium determined after separation of the metabolites by HPLC. Experiments were performed twice and the figure represents a single experiment.

FIGURES 7A-7B. Effect of bis(POM)PMEA concentration on PMEA efflux. Both CEM-SS and CEM-r1 cells were incubated with increasing concentrations of the prodrug bis(POM)PMEA (range from 9.97 mM to 8.8 mM). After incubation, cells were pelleted and resuspended in drug-free medium and the amount of PMEA effluxing into the medium determined. Data are the mean from two separate experiments.

FIGURES 8A-8B. Southern Blot analysis. Genomic DNA was isolated from CEM-SS and CEM-r1 cells as described herein. EcoR I, Hind III, BamH I was used digest the DNA from each cell line. 10 μg of the restricted digest was size

fractionated on a 0.8% agarose gel and transferred to a charge modified nylon membrane. Hybridization was performed using ^{32}P-labeled probes specific for MRP1, MRP2, MRP3 and MRP4 as described herein.

FIGURE 9. RNAse protection assay. Total cellular RNA was isolated from CEM-SS and CEM-r1 cells. This RNA was then hybridized at 42°C with ^{32}P-labeled antisense RNA probe (359 bp) specific for MRP4 and processed as described herein. The resulting protected mRNA fragment was then resolved on a standard sequencing gel.

FIGURE 10. Hydrophilic domains in the C-terminus of MRP4 (SEQ. ID. NO.: 3) predict potential sites for MRP4 peptide synthesis.

FIGURE 11. Multiple sequence alignment of C-terminus of MRP family members permits the identification of possible unique MRP4 peptide fragments for antisera development. MRP1 (SEQ.ID.NO.: 21), MRP2 (CMOAT) (SEQ.ID.NO.: 22), MRP3 (SEQ.ID.NO.: 23), MRP5 (SEQ.ID.NO.: 24), MRP6 (SEQ.ID.NO.: 25), MRP4 (38091aa) (SEQ.ID.NO.:3).

FIGURE 12. GenBank databank analysis identifies that the peptide SGR LKE YDE PYV LLQ NKE SL (SEQ ID NO.:4) is potentially useful for anti-sera development.

FIGURES 13A-13B. Verification that MRP4 anti-sera detects bacterially expressed MRP4 and that CEMr1 cells specifically overexpress MRP4. Lysates from bacteria containing either the MRP4 protein or not were lysed and fractionated on denaturing polyacrylamide gels. The fractionated protein was transferred to nitrocellulose membranes and (A) probed with streptavidin alkaline phosphatase or (b) MRP4 antisera

FIGURE 14. The CEM-r1 cells specifically overexpress MRP4 protein and not MRP1 as revealed by Western blot analysis of membrane proteins. R=50^{MT} cells.

S=CEM-SS cells.

FIGURE 15. The MRP4 EST (Genbank Accession R35798) was entirely sequenced (SEQ ID NO.: 10) and found to contain an open reading frame of 171 amino acids representing the C-terminus of MRP4.

FIGURE 16. The MRP4 EST (Genbank Accession R35798) was translated in the three forward reading frames to identify the longest open reading frame (ORF). The two additional sequences show divergence from other published sequence ORF fragments from the same clone.

FIGURE 17. Alignment between sequenced MRP4 regions reveals a region of sequence divergence (SEQ ID NO.: 3), (SEQ ID NO.:12).

FIGURE 18. Scheme for development of a MRP4 PCR assay utilizing an internal control MRP4 plasmid containing a 65bp deletion. Primer 9 and 3' RACE are used to delete 65 bp from MRP4 plasmid, resulting in a plasmid which is useful as an internal control.

FIGURES 19A-19C. Use of a degenerate oligonucleotide strategy to obtain a much larger portion of the MRP4 cDNA. The conserved VGRTGAKSS sequence is used to generate degenerate oligonucleotides. (A) The multiple sequence alignment shows among the p-glycoprotein family members the conservation of the Walker A motif (G(X4)GKS) (SEQ ID NO.: 13). (B) The multiple sequence alignment of the ORFs of the MRP family members reveals that MRP4 does not contain a Walker A while other MRP ESTs contain the Walker A. (C) Result of PCR amplification using walker A and MRP4 specific oligonucleotides of the MRP4 product from CEM-r1.

FIGURE 20. Sequence analysis reveals the presence of Walker A in MRP4 amplified sequence. (SEQ ID NO.: 14). The position of the Walker A motif is at

the 5' end region of the 2.7 kb fragment.

FIGURE 21. A Genbank database search reveals that the MRP4 sequence is highly related to another MRP family member, cMOAT (aka, MRP2) (SEQ ID NO.: 15).

FIGURE 22. MPR4 Is Abundantly Expressed In Many Tumor Cells.

FIGURE 23. MRP4 Expression Is Down-Regulated By p53 In K562 Erytholeukemia Cells.

FIGURE 24. [^{3}H]-PMEA is Preferentially Sorted to the Basal Compartment in Caco-02 Cells; Basal Flux is Inhibited by Prostaglandin A.

FIGURES 25A-C. PMEA Resistant CEM Cell Lines Have Increased MRP4 Gene Copy and Expression. (A) Southern blot, (B) RNAse protection assay, (C) Western Blot.

FIGURE 26A. Southern Blot analysis demonstrates specific amplification of MRP4. (A) Genomic DNA (10 ug) isolated from CEMss (S) and CEM-r1 cells (R) was digested with the indicated restriction enzymes followed by Southern blotting and sequential hybridization with ^{32}P-labeled cDNA probes MRP1, MRP2/cMOAT MRP3, and MRP4. Hybridized bands were visualized autoradiographically and quantified by densitometry.

FIGURE 26B. Cytogenetic evidence for MRP4 amplification in CEM-r1 cells. The MRP4 cDNA was used to screen a human leukocyte genomic library (Clontech). One MRP4 genomic clone containing intron sequence and exonic sequences homologous to the MRP4 cDNA was purified, sequenced, and used for fluorescent in situ hybridization analysis. In normal lymphocytes (CEMss) MRP4 specifically hybridized to human chromosome 13q32. To confirm chromosomal localization a probe (Oncor) that recognizes the centromere from chromosome 21 and 13 was used. Of 50 independent CEM-r1 cells

examined, 46 had the MRP4 homogeneous staining region with the same number of copies of MRP4 gene, and they were all observed at the distal arm of chromosome 13q.

FIGURE 27. MRP4 overexpression in CEM-r1 cells. (A) Lysates from BL21 bacteria or BL21 transformed with MRP4 in frame (MRP4) were analyzed on immunoblots. The fusion protein was localized with strepavidin alkaline phosphatase detection (not shown) or with affinity purified peptide antiserum against human MRP4. (B) CEMss and CEM-r1 cells were lysed in buffer containing a protease inhibitor cocktail (Boehinger Mannheim) and analyzed on immunoblots with either MRP4 or MRP1 antiserum (monoclonal PRL1 (Oncogene Science) reacted with peroxidase-conjugated antibodies and detected by enhanced chemiluminescence (Amersham).

FIGURE 28. Amplification and overexpression of MRP4 in CEMss cells selected for PMEA resistance. Individual PMEA resistant CEM cell lines were developed from CEMss cells by step-wise to exposure to increasing concentrations of 0.4, 2, 10 and 50 mM PMEA. (A) Southern blot analysis was performed on genomic DNA (10 ug) isolated from the indicated PMEA resistant cell lines and hybridized with MRP4. (B) 10 ug of total RNA from the same cell lines were analyzed by RNAse protection. (C) Immunoblot analysis of MRP4 and MRP1 was performed on total cell lysates of the same cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an isolated nucleic acid encoding a multi-drug resistance protein 4 (MRP4) or a portion thereof. An embodiment according to this invention is an isolated multi-drug resistance protein 4 (MRP4) nucleic acid having the following characteristics: (1) encoding an MRP4 protein; and (2) the ability to hybridize under standard hybridization conditions to the sequence shown in SEQ.

ID.No.:1 or a portion thereof. An embodiment of the present invention is the provided nucleic acid comprising SEQ.ID.No.:1 or a portion thereof.

According to an embodiment of the present invention the nucleic acid is selected from the group consisting of DNA, RNA and cDNA. Another embodiment of the present invention is a vector comprising the provided nucleic acid. According to still another embodiment of this invention, the vector comprises viral or plasmid DNA. Still another embodiment of the present invention is an expression vector comprising the provided nucleic acid and regulatory elements. An additional embodiment of the present invention provides a host vector system which comprises the provided expression vector in a suitable host. The present invention additionally provides a vector, comprising cDNA encoding MRP4 (ATCC Accession No.:_____). According to yet another embodiment of the present invention the suitable host is selected from the group consisting of a bacterial cell, a eukaryotic cell, a mammalian cell and an insect cell.

The present invention also provides an isolated MRP4 protein or a portion thereof. According to one embodiment of the present invention, MRP4 has substantially the same amino acid sequence as shown in SEQ.ID.No.: 2.

The present invention additionally provides a nucleic acid probe capable of specifically hybridizing with the provided nucleic acid. According to an embodiment of this invention, the probe is capable of specifically hybridizing with a nucleic acid selected from the group consisting of SEQ.ID.No:5, SEQ.ID.No:6, SEQ.ID.No:7, and SEQ.ID.No:8.

Also, the present invention provides an antibody capable of specifically binding to the provided protein without substantially cross-reacting with non-MRP4 proteins or homologs thereof under conditions permissive to antibody binding. An embodiment of this invention is a cell capable of producing the provided antibody. Additionally, the present invention provides a method of identifying the provided

MRP4 protein in a sample comprising: a) contacting the sample with the provided antibody under conditions permissive to the formation of a complex between the antibody and the protein; b) determining the amount of complex formed; and c) comparing the amount of complex formed in step (b) with the amount of complex formed in the absence of the antibody, the presence of an increased amount of complex formed in the presence of the antibody indicating identification of the protein in the sample.

The present invention further provides a method for identifying a nucleic acid in a sample which encodes MRP4 protein which comprises: (a) contacting the sample with the provided nucleic acid probe under conditions permissive to the formation of a complex between the nucleic acid probe and the nucleic acid encoding the MRP4 protein in the sample; (b) determining the amount of complex formed in step (a); and (c) comparing the amount of complex determined in step (b) with the amount of complex formed using an arbitrary sequence, a greater amount of complex formed with the MRP4-specific probe indicating the presence of a nucleic acid encoding a MRP4 protein in the sample. An embodiment of the present invention is further comprising amplifying the nucleic acid molecule encoding the MRP4 protein under conditions suitable for polymerase chain reaction. According to yet another embodiment of this invention, the amplified nucleic acid molecule encoding MRP4 is detected by size fractionation. According to still another embodiment, the probe is labeled with a detectable marker. According to a further embodiment, the detectable marker is a radiolabeled molecule, a fluorescent molecule, an enzyme. a ligand, or a magnetic bead. Still further, an embodiment of this invention provides an isolated nucleic acid, previously unknown, identified by the provided method.

Still further, the present invention provides a method for identifying a compound that modulates expression of MRP4 comprising: (a) contacting a sample which expresses MRP4 with the compound; (b) determining the amount of expression of MRP4 protein in the sample; and (c) comparing the amount of MRP4 protein expression determined in step (b) with the amount determined in the absence of the

compound. Also the present invention provides a compound, previously unknown, identified by the provided method.

According to an embodiment of the present invention the sample is selected from the group consisting of cell lysate, a cell-free translation expression system, an isolated cell and a cultured host cell.

Further still, the present invention provides a method for identifying a compound capable of modulating MRP4 protein activity comprising: (a) contacting a sample which expresses MRP4 protein with the compound; (b) determining the amount of MRP4 protein activity in the sample; and (c) comparing the amount of MRP4 protein activity determined in step (b) with the amount determined in the absence of the compound, an increase or decrease in activity indicating the presence of a compound capable of modulating the MRP4 protein activity. An embodiment of this invention is step (a) comprising first introducing the nucleic acid encoding a MRP4 protein into an expression system and causing the expression system to express the nucleic acid under conditions whereby a MRP4 protein is produced. According to a further embodiment of this invention, step (b) comprises measuring the efflux of an antimicrobial agent from a cell in the presence of the compound. According to a still further embodiment, the agent is selected from the group consisting of nucleoside inhibitors and protease inhibitors. According to yet a further embodiment, the agent is selected from the group consisting of antiviral agents such as azidothymidine (AZT), acyclovir and gancyclovir. According to yet another embodiment, the agent is selected from the group consisting of anti-neoplastic agents, such as AraC, Adenine arabinoside (Ara-A), 2-Chlorodeoxyadenosine (2-CDA), 2-fluoroarabinosyladenine and gemcitabine. The present invention further provides a compound, previously unknown, identified by the provided method.

According to an embodiment of the present invention the sample is selected from the group consisting of cell lysate, a cell-free translation expression system, an isolated

cell and a cultured cell. According to still yet another embodiment, the compound is a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule. According to still yet a further embodiment, the compound is bound to a solid support.

The present invention still further provides a method of modulating MRP4 protein activity in a sample, comprising contacting the sample with the compound identified by the provided method.

The present invention yet still further provides a pharmaceutical composition which comprises the identified compound and a pharmaceutically acceptable carrier. According to an embodiment of this invention, the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

Still further, the present invention provides a method for treating a condition in a subject which comprises administering to the subject an amount of the provided pharmaceutical composition, effective to treat the condition in the subject. According to an embodiment of this invention, the condition is selected from the group consisting of an infectious, immunodeficiency, neurological, renal, pulmonary, hepatic, cardiovascular, neoplastic and malignant condition. According to still a further embodiment of this invention, the condition is a result of virus, bacterial, or yeast infection.

Further still, the present invention provides a method for identifying subjects at risk for resistance to anti-microbial agent therapy comprising: (a) identifying by the provided method, the presence of MRP4 in a sample from the subject; (b) measuring the amount of MRP4 present in the sample from the subject; (c) comparing the amount of MRP4 present in a control sample having an amount of MRP4 which does not indicate resistance to drug therapy, an elevated amount of MRP4 present in the sample from the subject indicating increased risk for resistance to drug therapy in the subject.

Yet further still, the present invention provides a method for identifying an anti-microbial agent which is refractive to MRP4 efflux activity comprising: (a) contacting a cell expressing MRP4 with the agent; (b) measuring the amount of agent in the cell; (c) incubating the cell with the agent; and (d) comparing the amount of agent in the cell before and after the incubation of step (c) no substantial decrease in the amount of the agent in the cell after the incubation of step (c) indicating an agent which is refractive to MRP4 efflux activity. An embodiment of this invention is step (a) further comprising labeling the agent with a detectable marker. According to another embodiment of this invention, the detectable marker is a radiolabeled molecule, a fluorescent molecule, an enzyme, a ligand, or a magnetic bead. The present invention also provides the agent identified by the provided method. Yet another embodiment of this invention is a pharmaceutical composition comprising the agent and a pharmaceutically acceptable carrier.

Even further still, the present invention provides a transgenic non-human animal whose somatic and germ cells contain and express a gene encoding MRP4 protein the gene having been introduced into the animal or an ancestor of the animal at an embryonic stage and wherein the gene may be operably linked to an inducible promoter element. According to one embodiment of this invention, the transgenic animal is a mouse. According to another embodiment of this invention, the gene encoding MRP4 is overexpressed. According to still another embodiment, the transgenic animal is a knockout, comprising a genetic mutation which substantially reduces expression of MRP protein under normal conditions. A still further embodiment of this invention is a cell isolated from the transgenic animal. Further even still, the present invention provides a method of identifying an antimicrobial agent which is refractive to MRP4 efflux comprising the steps of: (a) contacting the cell isolated from the transgenic animal with the agent; (b) measuring the amount of agent in the cell; (c) incubating the cell with the agent; (d) comparing the amount of agent in the cell before and after the incubation of step (c) no substantial decrease in the amount of the agent in the cell after the incubation of step

(c) indicating a agent which is refractive to MRP4 efflux. According to yet still another embodiment of this invention, the transgenic animal overexpresses MRP4 protein.

Finally, the present invention provides a kit for identifying a compound which is refractive to MRP4 efflux comprising the provided probe. Also the present invention provides a diagnostic kit for identifying individuals resistant to anti-retroviral agent therapy comprising the provided probe, and/or antibody. According to an embodiment of this invention, the diagnostic kit further comprises nucleoside analogs or antibodies.

Antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the production or activity of MRP4 and/or subunits thereof, may possess certain diagnostic applications and may for example, be utilized for the purpose of detecting and/or measuring the susceptibility to or presence of MDR in cells or individuals, or with respect to drug refractivity to resistance development.

For example, MRP4 or any subunits thereof, may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Likewise, small molecules that mimic the activity(ies) of MRP4, may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols.

The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, *e.g.*, M. Schreier et al., "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies And T-cell Hybridomas" (1981); Kennett et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917;

4,472,500; 4,491,632; 4,493,890.

Panels of monoclonal antibodies produced against MRP4 or subunits thereof can be screened for various properties; *i.e.*, isotope, epitope, affinity, etc. Of particular interest are monoclonal antibodies that neutralize the activity of MRP4. Such monoclonals can be readily identified in MRP4 activity assays. High affinity antibodies are also useful when immunoaffinity purification of native or recombinant MRP4 is possible.

Preferably, the anti-MRP4 antibody used in the diagnostic methods of this invention is an affinity purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, it is preferable for the anti-antibody molecules used herein be in the form of Fab, Fab', $F(ab')_2$ or F(v) portions of whole antibody molecules.

One diagnostic method of the present invention comprises examining a cellular sample or medium by means of an assay including an effective amount of an antagonist to a MRP4 protein, such as an anti-MRP4 antibody, preferably an affinity-purified polyclonal antibody, and more preferably a mAb. In addition, it is preferable for the anti-MRP4 antibody molecules used herein be in the form of Fab, Fab', $F(ab')_2$ or F(v) portions or whole antibody molecules. Patients capable of benefitting from this method include those suffering from microbial infections such as viral infections and their related disorders such as Acquired Immune Deficiency in the case of human immunodeficiency virus (HIV); and cancers and their related disorders. Methods for isolating MRP4 and inducing anti-MRP4 antibodies and for determining and optimizing the ability of anti-MRP4 antibodies to assist in the examination of the target cells are all well-known in the art.

Methods for producing polyclonal anti-polypeptide antibodies are well-known in the art. See U.S. Patent No. 4,493,795 to Nestor et al. A monoclonal antibody, typically containing Fab and/or $F(ab')_2$ portions of useful antibody molecules, can be prepared

using the hybridoma technology described in *Antibodies - A Laboratory Manual*, Harlow and Lane, eds., Cold Spring Harbor Laboratory, New York (1988), which is incorporated herein by reference. Briefly, to form the hybridoma from which the monoclonal antibody composition is produced, a myeloma or other self-perpetuating cell line is fused with lymphocytes obtained from the spleen of a mammal hyperimmunized with a MRP4-binding portion thereof, or MRP4, or an origin-specific DNA-binding portion thereof.

Splenocytes are typically fused with myeloma cells using polyethylene glycol (PEG) 6000. Fused hybrids are selected by their sensitivity to HAT. Hybridomas producing a monoclonal antibody useful in practicing this invention are identified by their ability to immunoreact with the present MRP4 and their ability to inhibit either specified MRP4 activity in target cells.

A monoclonal antibody useful in practicing the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes antibody molecules of the appropriate antigen specificity. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well-known techniques.

Media useful for the preparation of these compositions are both well-known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al., *Virol.* **8**:396 (1959)) supplemented with 4.5 gm/l glucose, 20 mm glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the Balb/c.

Methods for producing monoclonal anti-MRP4 antibodies antibodies are also well-known in the art. See Niman et al., *Proc. Natl. Acad. Sci. USA*, **80**:4949-4953 (1983).

Typically, the present MRP4 or a peptide analog is used either alone or conjugated to an immunogenic carrier, as the immunogen in the before described procedure for producing anti-MRP4 monoclonal antibodies. The hybridomas are screened for the ability to produce an anti-MRP4 antibody that immunoreacts with the a peptide analog of MRP4 and the present MRP4.

Furthermore, the present invention relates to a variety of diagnostic applications and methods for detecting a susceptibility to MDR, a predisposition to MDR, or the presence of MRP4, or disorders related thereto, in relation to levels of MRP4 present in a patient as compared to a standard or control. Anti-MRP4 antibodies as described above, have broad applications in these types of diagnostic applications and methods.

As described in detail above, antibody(ies) to MRP4 can be produced and isolated by standard methods including the well known hybridoma techniques. For convenience, the antibody(ies) to MRP4 will be referred to herein as Ab_1 and antibody(ies) raised in another species as Ab_2 (secondary antibody).

The presence of MRP4 in cells can be ascertained by the usual immunological procedures applicable to such determinations. A number of useful procedures are known. Three such procedures which are especially useful utilize either the MRP4 labeled with a detectable label, antibody Ab_1 labeled with a detectable label, or antibody Ab_2 labeled with a detectable label. The procedures may be summarized by the following equations wherein the asterisk indicates that the particle is labeled, and "MRP4" stands for MRP4:

A. MRP4* + Ab1 = MRP4*Ab_1

B. MRP4 + Ab* = MRP4Ab_1*

C. MRP4 + Ab_1 + Ab_2* = MRP4Ab_1Ab_2*

The procedures and their application are all familiar to those skilled in the art and accordingly may be utilized within the scope of the present invention. The "competitive" procedure, Procedure A, is described in U.S. Patent Nos. 3,654,090 and 3,850,752. Procedure C, the "sandwich" procedure, is described in U.S. Patent Nos.

RE 31,006 and 4,016,043. Still other procedures are known such as the "double antibody," or "DASP" procedure.

In each instance, the MRP4 forms complexes with one or more antibody(ies) or binding partners and one member of the complex is labeled with a detectable label. The fact that a complex has formed and, if desired, the amount thereof, can be determined by known methods applicable to the detection of labels.

It will be seen from the above, that a characteristic property of Ab_2 is that it will react with Ab_1. This is because Ab_1 raised in one mammalian species has been used in another species as an antigen to raise the antibody Ab_2. For example, Ab_2 may be raised in goats using rabbit antibodies as antigens. Ab_2 therefore would be anti-rabbit antibody raised in goats. For purposes of this description and claims, Ab_1 will be referred to as a primary or anti-MRP4 antibody, and Ab_2 will be referred to as a secondary or anti-Ab_1 antibody.

The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

MRP4 or its binding partner(s) can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re.

Enzyme labels are likewise useful, and can be detected by any of the presently utilized calorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction

with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, ß-glucuronidase, ß-D-glucosidase, ß-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

A particular assay system developed and utilized in accordance with the present invention, is known as a receptor assay. In a receptor assay, the material to be assayed is appropriately labeled and then certain cellular test colonies are inoculated with a quantity of both the labeled and unlabeled material after which binding studies are conducted to determine the extent to which the labeled material binds to the cell receptors. In this way, differences in affinity between materials can be ascertained.

Accordingly, a purified quantity of MRP4 may be radiolabeled and combined, for example, with antibodies or other inhibitors thereto, after which binding studies would be carried out. Solutions would then be prepared that contain various quantities of labeled and unlabeled uncombined MRP4, and cell samples would then be inoculated and thereafter incubated. The resulting cell monolayers are then washed, solubilized and then counted in a gamma counter for a length of time sufficient to yield a standard error of <5%. These data are then subjected to Scatchard analysis after which observations and conclusions regarding material activity can be drawn. While the foregoing is exemplary, it illustrates the manner in which the assay may be performed and utilized.

A PCR assay can be also utilized. For example, for detection of MRP4 nucleic acid, the following primers may be used: SEQ ID NO: 1 or portions thereof, SEQ ID NO: 5 and SEQ ID NO: 6). PCR is generally performed as described, using the thermostable enzyme Taq polymerase (1.5u/sample) (AmpliTaq, Perkin Elmer Cetus) and a programmable PCR apparatus (MJ Research, Inc.). Target sequences are amplified as described herein and may be electrophoresed in a 6% 1 X TBE

polyacrylamide gel, at 1,200Vx2.5h at room temperature, gels dried and signal detected by overnight autoradiography with or without an intensifying screen.

Commercial test kits suitable for use by a medical or laboratory specialist may be prepared to determine the presence or absence of MRP4 activity in a test sample or in a predetermined (control) sample, or the presence of MRP4 protein or subunits thereof in a test sample or in a predetermined (control sample), or the presence of MRP4 encoding nucleic acid in a test sample or in a predetermined (control sample), or the presence of nucleic acid capable of binding MRP4 encoding nucleic acid or subunits thereof in a test sample or in a predetermined (control) sample.

In accordance with the testing techniques discussed above, one class of such kits will contain at least labeled MRP4 or its binding partner, for instance an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., "competitive," "sandwich," "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Another class of such kits may also include PCR reagents, such as oligonucleotide primers, enzymes, gel matrixes, buffers, etc.

Accordingly, a test kit may be prepared for the diagnosis or detection of a susceptibility or predisposition to MDR related conditions including those resulting from infectious diseases and cancers.

Such a kit may be comprising: (a) a predetermined amount of at least one labeled immunochemically reactive component obtained by the direct or indirect attachment of present MRP4 factor or a specific binding partner thereto, to a detectable label; (b) other reagents; and (c) directions, including comparison levels of MRP4, for use of said kit.

An alternate kit for measuring the levels of MRP4 activity may comprise PCR

reagents, such as oligonucleotide primers, enzymes, gel matrixes, buffers, directions, including comparison levels of MRP4, for use of said kit. A still further alternate can utilize reagents for measuring the levels of MRP4 activity as described; and directions, including comparison levels of MRP4, for use of said kit. More specifically, the diagnostic test kit may comprise: (a) a known amount of the MRP4 or fragment thereof as described above (or a binding partner) generally bound to a solid phase to form an immunosorbent, or in the alternative, bound to a suitable tag, or plural such end products, etc. (or their binding partners) one of each; (b) if necessary, other reagents; and (c) directions, including comparison levels of MRP4, for use of said kit.

In a further variation, the test kit may be prepared and used for the purposes stated above, which operates according to a predetermined protocol (e.g. "competitive," "sandwich," "double antibody," etc.), and comprises: (a) a labeled component which has been obtained by coupling the MRP4 to a detectable label; (b) one or more additional immunochemical reagents of which at least one reagent is a ligand or an immobilized ligand, which ligand is selected from the group consisting of: (i) a ligand capable of binding with the labeled component (a); (ii) a ligand capable of binding with a binding partner of the labeled component (a); (iii) a ligand capable of binding with at least one of the component(s) to be determined; and (iv) a ligand capable of binding with at least one of the binding partners of at least one of the component(s) to be determined; and (c) directions for the performance of a protocol for the detection and/or determination of one or more components of an immunochemical reaction between the MRP4 and a specific binding partner thereto.

In accordance with the above, an assay system for screening potential drugs effective to modulate (*i.e.* increase or decrease) the levels of, or the activity of, the MRP4 may be prepared. The MRP4 may be introduced into a test system, and the prospective drug may also be introduced into the resulting cell culture, and the culture thereafter examined to observe any changes in the MRP4 activity of the cells, due either to the addition of the prospective drug alone, or due to the effect of added quantities of the

known MRP4.

In addition to PCR based diagnostics, more traditional hybridization techniques may be used based on well known methods using MRP4-conserved sequences which show homology to MRP4 or sequences which specifically hybridize with MRP4. Nucleic acid analogs are also contemplated.

As used herein, a sequence is conserved if there is substantial homology of sequence between multiple gene species.

As used herein, the terms, "hybridization" and "binding" in the context of probes, primers and denatured DNA are used interchangeably. Probes which are hybridized or bound to denatured DNA are aggregated to complementary sequences in the polynucleotide. Whether or not a particular probe remains aggregated with the polynucleotide depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must the degree of complementarity, and/or the longer the probe.

As used herein, the terms, "probe" and "primer" refer to an oligonucleotide designed to be sufficiently complementary to a sequence in a denatured nucleic acid to be probed, in relation to its length, to be bound under selected stringency conditions. The terms probes and primers may be used interchangeably. Probes may vary in length. Preferably such probes should be sufficiently long to hybridize to the modified RNAs in a specific and stable manner. A "semi-random probe" as the term is used herein, encompasses a class of probes wherein either a discrete portion of the probe is random, while another discrete portion is conserved as well as probes which have nucleotide preferences at particular positions within a sequence. For example, the discrete portion-type probe may have a predetermined adaptor sequence at its 5' end and a random sequence at its 3' end. Alternatively, several preferred probes have nucleotide preferences at specific positions within the probes while other positions are random. A "degenerate probe" as the term is used herein, encompasses a cocktail or

mixture of probes wherein one or more of the possible triplet nucleotide sequences encoding an amino acid is incorporated into the probe sequence. For example, Serine may be encoded by six separate triple sequences (AGU, AGC, UCU, UCC, UCA, and UCG). Thus, a degenerate probe may reflect the degeneracy of the nucleotide triplet code. Alternatively, a randomized probe, as the term is used herein, encompasses a probe wherein the nucleotide at one or more positions may be randomized in order to yield a triplet sequence encoding an alternative or a random amino acid at the position.

An "end region" as the term is used herein, consists of the end nucleotide and a portion of the region including as much as that half of the entire sequence. For example, the "3' end region" or "3' region" of a probe may include the 3' half of the probe.

A preferred method of hybridization is blot hybridization. See Sambrook et al. 1989 *Molecular Cloning: A Laboratory Manual* 2nd Ed. for additional details regarding blot hybridization. Using this method, separated amplification products are transferred onto a solid matrix, such as a filter. The probe, which is detectable, either directly or indirectly, is hybridized to the solid matrix under appropriate conditions. The matrix is washed to remove excess probe. Thereafter the probe which specifically hybridized to the solid matrix can be detected.

The probe can be DNA or RNA and can be made detectable by any of the many labeling techniques readily available and known to the skilled artisan. Such methods include, but are not limited to, radio-labeling, digoxygenin-labeling, and biotin-labeling. A well-known method of labeling DNA is ^{32}P using DNA polymerase, Klenow enzyme or polynucleotide kinase. In addition, there are known non-radioactive techniques for signal amplification including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R.N.K. et al, 1973 *Proc. Natl. Acad. Sci. USA* 70:2238-42), methods which allow detection by chemiluminescence (Barton, S.K. et al, 1992 *J. Am. Chem. Soc.* 114:8736-40) and methods utilizing

biotinylated nucleic acid probes (Johnson, T.K. et al, 1983 *Anal. Biochem.* 133:125-131; Erickson, P.F. et al, 1982 *J. Immunol. Methods* 51:241-49; Matthaei, F.S. et al, 1986 *Anal. Biochem.* 157-123-28) and methods which allow detection by fluorescence using commercially available products. Non-radioactive labeling kits are also commercially available.

A basic description of nucleic acid amplification is described in Mullis, U.S. Patent No. 4,683,202, which is incorporated herein by reference. The amplification reaction uses a template nucleic acid contained in a sample, two primer sequences and inducing agents. The extension product of one primer when hybridized to the second primer becomes a template for the production of a complementary extension product and vice versa, and the process is repeated as often as is necessary to produce a detectable amount of the sequence.

The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E.coli* DNA polymerase I, thermostable *Taq* DNA polymerase, Klenow fragment of *E.coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase and other enzymes which will facilitate combination of the nucleotides in the proper manner to form amplification products. The oligonucleotide primers can be synthesized by automated instruments sold by a variety of manufacturers or can be commercially prepared based upon the nucleic acid sequence of this invention.

The degree of hybridization depends on the degree of complementarity, the length of the nucleic acid molecules being hybridized, and the stringency of the conditions in a reaction mixture. Stringency conditions are affected by a variety of factors including, but not limited to temperature, salt concentration, concentration of the nucleic acids, length of the nucleic acids, sequence of the nucleic acids and viscosity of the reaction mixture. More stringent conditions require greater complementarity between the nucleic acids in order to achieve effective hybridization.

Solid matrices, useful for hybridization or binding assays or in diagnostic kits are available to the skilled artisan. Solid phases useful to serve as a matrix for the present invention include but are not limited to polystyrene, polyethylene, polypropylene, polycarbonate, or any solid plastic material in the shape of test tubes, beads, microparticles, dip-sticks, plates or the like. Additionally matrices include, but are not limited to membranes, 96-well microtiter plates, test tubes and Eppendorf tubes. Solid phases also include glass beads, glass test tubes and any other appropriate shape made of glass. A functionalized solid phase such as plastic or glass which has been modified so that the surface carries carboxyl, amino, hydrazide, or aldehyde groups can also be used. In general such matrices comprise any surface wherein a ligand-binding agent can be attached or a surface which itself provides a ligand attachment site.

As used herein, "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. A pharmaceutically acceptable carrier encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. The invention also provides for pharmaceutical compositions together with suitable diluents, preservatives, solubilizers, emulsifiers and adjuvants. Other embodiments of the compositions of the invention incorporate particulate forms, protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including but not limited to intravenous, intramuscular, parenteral, pulmonary, nasal and oral.

As used herein, an "effective amount" is the amount required to achieve a clinically significant reduction in infection, preferably of at least 30 percent, more preferably of at least 50 percent, most preferably of at least 90 percent. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated. For the purposes of this invention, the methods of administration are to include, but are not limited to administration cutaneously, subcutaneously, intravenously, parenterally, orally, topically, or by aerosol.

The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in admixture, a pharmaceutically acceptable excipient (carrier) and one or more of a polypeptide analog or fragment of the provided peptide or peptide composition, a peptidomimetic composition thereof as described herein as an active ingredient. A cocktail of the provided pharmaceutical composition in various combinations is also contemplated.

The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

A polypeptide, analog or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of

the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The therapeutic polypeptide-, analog- or active fragment-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; *i.e.*, carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

As used herein, the term "synthetic amino acid" means an amino acid which is chemically synthesized and is not one of the 20 amino acids naturally occurring in nature. As used herein, the term "biosynthetic amino acid" means an amino acid found in nature other than the 20 amino acids commonly described and understood in the art as "natural amino acids."

As used herein, amino acid residues are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. Abbreviations for amino acid residues are used in keeping with standard polypeptide nomenclature delineated in *J. Biol. Chem.*, 243:3552-59 (1969).

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues.

Amino acids with nonpolar R groups include: Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan and Methionine. Amino acids with uncharged polar R groups include: Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine and Glutamine. Amino acids with charged polar R groups (negatively charged at Ph 6.0) include: Aspartic acid and Glutamic acid. Basic amino acids (positively charged at pH 6.0) include: Lysine, Arginine and Histidine (at pH 6.0). Amino acids with phenyl groups include: Phenylalanine, Tryptophan and Tyrosine. Particularly preferred substitutions are: Lys for Arg and vice versa such that a positive charge may be maintained; Glu for Asp and vice versa such that a negative charge may be maintained; Ser for Thr such that a free -OH can be maintained; and Gln for Asn such that a free NH_2 can be maintained. Amino acids can be in the "D" or "L"

configuration. Use of peptidomimetics may involve the incorporation of a non-amino acid residue with non-amide linkages at a given position.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (*i.e.*, His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces -turns in the protein's structure.

As used herein, "pM" means picomolar. "nM" means nanmolar, "μM, means micromolar, "mM" means millimolar, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter. As used herein, "ED_{50}", the effective dose 50, means the drug concentration yielding 1/2 maximal RT activity. "IC_{50}" means the inhibitory concentration at which growth is arrested by 50 percent.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention. While the invention is described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

EXAMPLES

EXAMPLE 1 CELL VARIANTS RESISTANT TO ANTI-VIRAL NUCLEOSIDE ANALOGS CONTAIN AN EFFLUX PUMP

In this example, a T lymphocytic cell line was selected for PMEA resistance following exposure to cytotoxic concentrations of PMEA. The results show that the variant cells,

termed CEM-r1, were limited in their ability to accumulate PMEA and its metabolites and exhibited enhanced cellular export of PMEAp. A modest alteration in phosphorylation of PMEA was also observed in the variant cells. Moreover, this resistant cell displays cross resistance to other related acyclic nucleoside phosphonate analogs including PMEG, AZT and 3TC. A second cell variant, resistant to PMEG, termed CEM-RPMEG, also displays cross-resistance to PMEA, AZT and 3TC. The results indicate that the multidrug resistance phenotypes are associated with an increased efflux of the active drug metabolite from the cells.

RESULTS

Selection of PMEA-resistant cells. Selection for PMEA resistance was carried out by exposing CEM-SS cells in culture to increasing concentrations of PMEA, over several months. Of the various lines that grew in the presence of high concentrations of PMEA, one (designated CEM-r1) was selected and cultured for at least 10 generations in PMEA-free medium before subsequent studies. CEM-r1 cells, cultured in the presence or absence of PMEA, were morphologically indistinguishable from the parent CEM-SS cells. However, as shown in Figure 1, CEM-r1 cells were ~250-fold more resistant to the cytotoxic effects of PMEA, compared with the parental cells. This drug resistance of CEM-rl cells remained stable for at least 3 months of cell culturing in the absence of PMEA.

Uptake and metabolism of [^{3}H]PMEA. Because PMEA requires activation to the diphosphorylated derivative PMEApp for its activity, it was determined whether the mutant cells exhibit altered drug anabolism. The mutant and parental cells were incubated with 10 μM [^{3}H]PMEA and at various times were analyzed for intracellular levels of PMEA and its metabolites by Partisil SAX HPLC. In the parental cells the accumulation of PMEA and its anabolites PMEAp and PMEApp was dependent on the concentration of PMEA in the external medium at least up to 2 mM, with no evidence of saturation in either the uptake or phosphorylation of the drug. The parental CEM-SS cells accumulated PMEA and its anabolites PMEAp and PMEApp in a relatively linear fashion for at least 100 min, during which

measurements were made. In contrast, CEM-r1 cells accumulated markedly lower levels of PMEA than did the parental cells and there were virtually no anabolites, PMEAp and PMEApp, detectable within this time period (Figure 2). The metabolism of [^{3}H]PMEA was also examined in both cell lines after longer periods (*i.e.*, 8 and 12 hr) of incubation with 10 μM [^{3}H]PMEA. As shown in Table 1 below, in wild-type cells intracellular PMEA levels measured after 8 and 12 hr reached 1.7 and 2.5 μM, respectively, and PMEAp and PMEApp concentrations increased to 0.23 and 0.41 μM, respectively, after a 12-hr incubation period. In contrast, PMEA levels in CEM-r1 cells after 12 hr reached only 1 μM (40% of the wildtype level) and PMEAp and PMEApp levels were only 2-3% of parental cell levels after 12 hr.

TABLE 1

Intracellular Accumulation Of PMEA, PMEAp And PMEApp In CEM-SS And CEM-R1 Cells

	Intracellular levels			
	CEM-SS		CEM-r1	
	8 hr	12hr	8 hr	12 hr
	μM			
PMEA	1.7	2.5	1.0 (56%)	0.99 (40%)
PMEAp	0.13	0.23	0.005 (3.8%)	0.005(2.2%)
PMEApp	0.22	0.41	0.008 (3.6%)	0.01 (2.4%)

Exponentially growing cultures were incubated with 10 μM [^{3}H] PMEA for 8 or 12 hr and the cell extracts were analyzed for PMEA and its metabolites by ion exchange HPLC, as described.

Uptake and metabolism of [^{3}H]bispom-PMEA. Lower levels of PMEA and anabolites in CEM-r1 cells could result from decreased uptake, increased excretion, and/or decreased activation of the drug. However, the level of radioactive PMEA accumulating in CEM-r1 cells was too low for a detailed analysis of the specific mechanism involved. Therefore, the metabolism of [^{3}H]bispom-PMEA (15), a lipophilic prodrug of PMEA, was analyzed in the two cell lines in order to further examine the mechanism of

resistance in the mutant cell line. It was previously shown that bispom-PMEA, unlike PMEA, is rapidly taken into cells and converted by cellular esterases to unmodified PMEA (24). Figure 2 shows data on the early time course (120 sec) of 1 μM [^{3}H]bispom-PMEA entry into the parental and CEMr1 cells. There was no significant difference between the two cell lines in their initial uptake of bispom-PMEA. However, when the cells were incubated for longer periods with [^{3}H]bispom-PMEA, significant differences in the accumulation of drug metabolites were observed. Table 2 depicts results from an experiment in which the two cell lines were incubated with l μM [^{3}H]bispom-PMEA for 15 min. As shown previously (24), the extent of accumulation of PMEA and its metabolites PMEAp and PMEApp in the parental CEM-SS cells was much higher after a 15-min incubation with 1 μM bispom-PMEA than after an 8-hr incubation with 10 μM PMEA (compare Tables 1 and 2). In the resistant CEM-rl cells, however, incubation with 1 μM [^{3}H]bispom-PMEA resulted in levels of tritiated PMEA, PMEAp, and PMEApp that were only about one third of those seen in parental cells. Only ~2% of the radioactivity present in the cells after incubation could be detected as the intact pro-drug and this was similar in parental and resistant cells, indicating that there was no significant difference in the hydrolysis of the pro-drug to the unmodified parental drug in the two cell lines.

TABLE 2

Metabolism Of [^{3}H]Bispom-PMEA In CEM-SS And CEM-r1 Cells

Metabolite	Metabolite Levels			
	Cells		Medium	
	CEM-SS	CEM-r1	CEM-SS	CEM-r1
	μM		nM	
PMEA	33.1	13.3	4.8	23.2
PMEAp	0.48	0.17	NDa	ND
PMEApp	0.16	0.07	ND	ND
Bispom-PMEA	0.03	0.04	490	429
Monopom-PMEA	4.6	4.1	185	166

a ND, not determined

Exponentially growing cultures were incubated with 1μM [^{3}H]bispom-PMEA for 15 minutes and the various metabolites in the cell extracts and media were analyzed by ion exchange HPLC, as described. Data are shown for one individual experiment; essentially similar results were obtained in a second independent experiment.

The results in Table 2 also include the levels of various metabolites in the medium of cells incubated with tritiated [^{3}H]bispom-PMEA. Most of the radioactivity remaining in the medium was associated with bispom-PMEA and monopomPMEA, and this was comparable for the two cell lines. However, a significant difference was seen in the levels of extracellular PMEA, which were ~5-fold higher with the mutant CEM-rl cells than with the parental CEM-SS cells. Control experiments revealed that under these conditions direct extracellular hydrolysis of bispom-PMEA in the medium did not contribute significantly to PMEA in the medium. Thus, this observation suggested that the appearance of PMEA in the medium was due to the excretion from cells of the drug derived from intracellular metabolism of bispom-PMEA.

Measurements of efflux of [^{3}H]PMEA. To examine whether the resistant cells could be altered in their capacity to retain the phosphonate analog, the clearance of [^{3}H]PMEA and its metabolites from CEM-SS and CEM-r1

cells was determined. CEM-SS or CEM-r1 cells were incubated for 15 minutes with 1μM or 2μM [^{3}H]bispom-PMEA, respectively, in an attempt to accumulate comparable levels of intracellular PMEA and anabolites, and were then incubated in drug-free medium. Aliquots of cells and medium sampled at different intervals over a period of 2 hr were analyzed by HPLC, and the results are shown in Figure 4. The intracellular levels of radioactivity associated with PMEA declined gradually in the parental cells, and half-maximal clearance was attained within about 60 min. Some of this clearance was probably due to the anabolism of PMEA, because the PMEAp- and PMEApp-associated radioactivity continued to accumulate within the cells, but the majority of the radioactivity was excreted into the medium as PMEA. In contrast to the results with parental cells, loss of intracellular PMEA-associated radioactivity in the resistant CEM-r1 cells was extremely rapid and the majority of the radioactivity within the cells was excreted into the medium within 20-30 minutes of incubation (Figure 4). From the increases in the extracellular radioactivity accumulated in the medium, one can calculate that the rate of efflux of PMEA was increased ~7-fold to 2.55 pmol/min/10^6 in resistant CEM-r1 cells, from 0.4 pmol/min/10^6 cells in parental CEM-SS cells.

Cross-resistance to other agents. The relative sensitivity of the wild-type and CEM-r1 cells to structurally related and unrelated analogs was also been examined. As shown in Table 3, the CEM-r1 cells are highly cross-resistant to the prodrug bispom-PMEA and the 2,6-diaminopurine derivative PMEDAP. Only partial cross-resistance was observed against two related nucleoside phosphonates, HPMPA and PMEG (Table 3). Interestingly, the mutant was also partially resistant to a number of unrelated purine nucleosides, including adenosine, 2-chlorodeoxyadenosine, and 2-fluoroarabinosyladenine, but not to arabinosyladenine, 2'-deoxyadenosine, hydroxyurea, or the adenine nucleotide precursor 5-amino-4-imidazolecarboxamide riboside. CEM-r1

cells were also not resistant to vinblastine and colchicine, two agents that are characteristic of the MDR phenotype in a number of mutant cell lines selected against these agents (25). It should be noted that the MDR mutant CEM/VLB_{100} (26) did not exhibit any cross-resistance to PMEA.

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TABLE 3

Comparative Resistance Of CEM And CEM-R1 Cells To Various Agents

Compound	IC_{50}		Relative Resistance
	CEM-SS	CEM-r1	
	μM		
PMEA	34	6200	182
PMEADAP	2.3	1200	533
Bispom-PMEA	0.26	32	123
PMEG	0.7	19	30
HPMPA	92	1100	12
Adenosine	38	205	5.4
2-Chlorodeoxyadenosine	0.08	0.4	5.5
2-Fluoroarabinosyladenine	1.2	4.4	3.7
Arabinosyladenine	0.7	0.9	~1
2'-Deoxyadenosine	1.7	2.5	~1
5-Amino-4-imidazolecarboxamide	130	106	~1
Hydroxyurea	55	50	~1
Vinblastine	0.0018	0.0021	~1
Colchicine	0.014	0.013	~1

The IC_{50} values were obtained after 2 days of culture. Each result is the mean of one representative experiment performed in duplicate. The cultures with the purine nucleosides adenosine, 2'-deoxyadenosine, and arabinosyladenine contained 5 μM deoxycoformycin to inhibit adenosine deaminase activity.

Enzyme levels. Cytosolic extracts of CEM-SS and CEM-r1 cells were also examined for differences in enzyme activity of potential importance for nucleoside/nucleotide analog metabolism. The two cell lines had
10 indistinguishable activities of adenosine kinase and deoxycytidine/deoxyadenosine kinase, enzymes that phosphorylate the

various purine nucleosides described above to their 5'-phosphorylated derivatives (27-30), and PRPP synthetase, which has been implicated in the phosphorylation of PMEA (11) (Table 4). However, the resistant CEM-rl cells did exhibit a 2-fold decrease in the activity of adenylate kinase, compared with the wild-type CEM-SS cells (Table 4), suggesting that this enzyme may be important for the phosphorylation of PMEA.

TABLE 4

Purine Nucleoside-Phosphorylating Enzymes In CEM-SS And CEM-R1 Cells.

Enzyme	Activity	
	CEM-SS	CEM-r1
	nmol/hr/mg of protein	
Adenosine kinase	143	168
Deoxycytidine/deoxyadenosine kinase	4.5	4.9
PRPP synthetase	492	480
Adenylate kinase	2675	1442

Mean values from at least two independent determinations are shown. The different enzyme activities, adenosine kinase (24), deoxycytidine kinase (24), PRPP synthetase (11), and adenylate kinase (25), were determined according to previously described procedures.

Antiviral activity and Cytotoxicity of AZT, 3TC, PMEA and PMEG in CEM-SS and CEM-rl cells. The PMEA-resistant cell line, CEM-rl, used in the present study was maintained in a drug-free medium for several months before studying the effects of PMEA, PMEG, AZT and 3TC. Table 5 summarizes the ED_{50} and the IC_{50} values, which indicate the antiviral activity and the cytotoxicity, respectively, in both the wild type and mutant cells.

Phosphorylation of AZT. Both CEM-SS and CEM-r1 cells were incubated with 10μM AZT and the phosphorylated biotransformation products of AZT present intracellularly or in the media were separated by solid phase extraction (19) and quantitated by liquid scintillation counting. The results, shown in Table 6, demonstrate a 4- to 5-fold decrease in the intracellular levels of AZTMP, AZTDP

and AZTTP in the CEM-rl cells compared with CEM-SS cells at the time periods evaluated. The appearance of the phosphorylated metabolites into the medium was also determined in both the cell lines. AZTDP and AZTTP were not detected in the medium of either CEM-SS or CEM-r1 cells. However, the amount of AZTMP accumulated in the media was approximately 3-fold higher in CEM-rl cells than in CEM-SS cells. The cellular integrity of both CEM-SS and CEM-rl cells was verified by Trypan Blue exclusion assay (22) throughout the time course of the experiments and was found to be greater than 98%.

TABLE 5

Antiviral Activity & Cytotoxicity of Analogs

DRUG	Antiviral Activity ED_{50} (μM)		Fold Resistance	Cytotoxicity IC_{50} (μM)		Fold Resistance
	WT	CEM-r1		WT	CEM-r1	
PMEA	2	>300	>150	80	20000	250
PMEG	-*	-*	-	5	200	40
AZT	0.006	0.140	23	500	3000	6
3TC	0.21	1.10	5.5	1000	5000	5

(*- PMEG did not have any antiviral activity against HIV)

The fold resistance for each drug was calculated as the ratio of the IC_{50} of the resistant cell line to the IC_{50} of the wild type cells for that drug.

Cellular Transport-Intracellular Accumulation of AZT: Using [^{3}H] AZT, the transport of AZT across the cellular membrane was studied in each cell line. Cellular uptake of AZT (10μM and 100μM) was measured up to 120 seconds and was similar in both the cell lines. The results displayed in Figure 5 indicate that both the CEM-SS and CEM-rl do not exhibit any deficiency in the uptake of the drug.

Cellular Transport: Efflux of AZT. Cells were incubated for 60 minutes with [^{3}H] -AZT (10 μM) after which they were washed free of radioactivity, resuspended

in drug-free medium and the intracellular and the extracellular concentration of [^{3}H]-AZT were measured. The results, displayed in Figure 6, show that following the removal of extracellular AZT the intracellular concentration of AZTMP decreased linearly over 16 minutes. At least 80% of the AZTMP present intracellularly in CEM-rl cells was excreted into the medium in 10 minutes (Figure 6A). The time ($t_{½}$) required to attain half the intracellular concentration of AZTMP compared to the intracellular concentration at the time of resuspension of cells in the drug-free medium was significantly different (CEM-SS: $t_{½}$ = 17.5 min.; CEM-rl:$t_{½}$ = 4.5 min.). However, there was less difference in the $t_{½}$ for intracellular AZT in the wild type and mutant cell lines (CEM-SS: $t_{½}$ = 8.1 min.; CEM-rl: $t_{½}$ = 6.5 min.). The intracellular levels of AZTDP and AZTTP, which represented 1% of total cellular anabolites, were also measured and did not change significantly in either of the two cell lines over the time period studied.

Effect of Concentration of Bis(POM)PMEA on the Efflux of PMEA. Both CEM-SS and CEM-rl cells were incubated for 3 minutes with increasing concentrations (range 0.07 mM to 8.80 mM) of the prodrug for the selecting agent, Bis(POM)PMEA. After incubation, the cells were pelleted and reconstituted in drug-free medium, and the amount of PMEA present in the medium and intracellularly was measured 15 minutes after resuspension. The amount of PMEA effluxing out into the medium at 15 minutes increased initially with increasing concentration (Figure 7A). At concentrations higher than 2.2 mM and 4.4 mM Bis(POM)PMEA, respectively, in CEM-SS and CEM-rl cells, the amount of PMEA effluxed out did not increase significantly indicating saturation of the protein-mediated efflux process at increasing concentrations of the drug. However, the amount of PMEA present intracellularly increased with increasing concentrations of the prodrug (see Figure 7B).

Effect of PMEA concentration gradient on PMEA efflux. CEM-r1 cells were incubated with 0.07-8.8mM Bis(POM)PMEA for 3 minutes. The cells were pelleted and reconstituted in either drug-free medium or in medium containing 0.5

mM PMEA, and the amount of PMEA present in the medium and intracellularly was measured 15 minutes after resuspension. The results, displayed in Figure 7A, show no significant difference in the amount of PMEA effluxed out in the presence or absence of PMEA in the medium, providing evidence for efflux of PMEA against a concentration gradient which is consistent with a protein-mediated efflux of the compound.

Inhibitors of Drug Efflux. To assess if the observed transport of AZTMP in CEM-rl cells was energy-mediated efflux, ATP was depleted from both of the cell lines by the addition of sodium azide. Both CEM-SS and CEM-rl cells were incubated in medium that contained 10 mM sodium azide and 10 mM 2-deoxy-D-glucose, and within 5 minutes the intracellular ATP levels (23) decreased by 90% but the viability of the cells was not altered.

Accordingly, in this example, the human T lymphoid cell line CEM-r1 was further examined as to its demonstration of efflux of acyclic nucleoside analogs such as PMEA and AZT. This cell line also displays cross resistance to other related acyclic nucleoside phosphonate analogs such as PMEG 9-(2-phosphonylmethoxyethyl)guanine) as well as the antiretroviral nucleoside analog AZT). Transport studies were conducted as set forth in Table 6 below, and indicated that the CEM-r1 cells rapidly effluxed PMEA and AZTMP (azido-thymidine monophosphate) but not their di- and triphosphate derivatives (not shown). Also, depletion of intracellular ATP by azide and 2-deoxyglucose caused a marked decrease in the rate of efflux of both PMEA and AZTMP in CEM-r1, but not from CEMss sensitive cells.

The P-glycoprotein (Pgp) antagonist, verapamil had no effect on the rate of efflux, however, a non-traditional Pgp antagonist dipyridamole [63] significantly decreased the rate of PMEA and AZTMP efflux (not shown). These studies suggested PMEA and AZTMP efflux occurred by an ABC cassette transporter that was unlikely to be P-glycoprotein because; i) verapamil had no effect on drug efflux and ii) Pgp was undetectable in CEMss

or CEM-r1 cells by Western blot analysis (not shown). Thus, it was hypothesized that another ABC cassette transporter was involved in PMEA and AZTMP efflux.

TABLE 6

Functional Characterization of CEMss and CEMr-1

EFFLUX KINETICS

	AZTMP (min)		PMEA (min)	
Treatment	CEMss	CEMr-1	CEMss	CEMr-1
None	17.5	4.3	>26	4.4
Azide + Deoxyglucose	16.3	18.1	>26	>26

DRUG SENSITIVITY (μM)

PMEA		AZT	
CEMss	CEMr-1	CEMss	CEMr-1
80	2000	500	3000

Referring to Table 6 in greater detail, efflux kinetics were studied. Transport analysis of PMEA was conducted as previously described [48]. Both the CEM-SS and CEM-rl cells were pre-labeled with [^{3}H]-AZT by incubation in its presence for 60 minutes. The cells were then washed free of radioactivity and then incubated in drug-free medium in the presence of sodium azide and 2-deoxy-D-glucose. At various time periods, the amount of intracellular AZTMP was measured. As reported in Table 6, depletion of ATP led to an inhibition of AZTMP efflux (*i.e.* a significant accumulation of AZTMP in the CEM-rl cells but not in the CEM-SS cells). The $t_{1/2}$, for AZTMP in energy-deprived CEM-rl cells ($t_{½}$ = 4 minutes) was almost totally restored to that observed in CEM-SS cells ($t_{½}$ = 17.1 min). Further,

depletion of cellular energy had very little effect on AZTMP efflux in CEM-SS cells ($t_{1/2}$ = 16.3 minutes in the presence of sodium azide and 2-deoxy-D-glucose). These findings suggest the presence of an active efflux system for AZTMP in the CEM-rl cells that does not appear to be expressed in the wild type cells, CEM-SS.

Drug Sensitivity: The cells were treated with varying concentrations of PMEA and AZT and the concentration to inhibit 50% growth after a 24 h exposure was determined as previously described [48].

RESULTS

The large magnitude and phenotypic stability of drug resistance for months in the absence of drug [48] of CEM-r1 cells suggested gene amplification might contribute to CEM-r1 drug resistance. Because of the recent expansion in the number of MRP related genes [49,61], genomic DNA from the CEMss and CEM-r1 cells was isolated, and was screened by Southern blot for changes in gene copy of genes encoding MRP1-4. The MRP1 gene was not amplified in the CEM-r1 cells (Fig 8), nor was MRP2 or MRP3 (not shown) as their signal intensity was not different between CEMss and CEM-r1 cells. In contrast, compared with CEMss, the CEM-r1 cell line had increased MRP4 gene copy, a finding consistent with gene amplification [62]. Densitometry revealed that the MRP4 gene was amplified from 8-10 fold. In addition, a longer exposure of the CEMss revealed that the MRP4 gene structure was no different in the CEM-r1 cells. To verify that the MRP4 gene was amplified, a MRP4 genomic clone was isolated and FISH analysis performed on metaphase chromosomes (Fig 26B). In CEMss cells MRP4 hybridized to chromosome 13 at position 13q32 (not shown) a more refined localization than previously reported [49]. In the CEM-r1 cells a large homogeneous staining region (HSR) was observed only on the distal arm of chromosome 13 (Fig 26B). This finding demonstrates that the increased MRP4 gene copy detected by Southern blot is due to gene amplification and not changes in CEM-r1 ploidy.

The compelling association between MRP4 amplification and the increased rate of drug

efflux is further substantiated by evidence that the MRP4 protein is overexpressed. Antiserum specific for MRP4 was developed and specificity was demonstrated by immunoblot detection of an in-frame MRP4 bacterial fusion (Fig 27A), but not the out of frame MRP4 (not shown), and the absence of MRP4 antiserum immunoreactivity with either Pgp/MDR1, MRP1, or MRP2 in lysates from cells expressing these proteins (not shown). Immunoblot analysis with anti-MRP4 antiserum revealed a dramatic overexpression of MRP4 in the CEM-r1 cells. The increase in MRP4 expression was specific because stripping and re-probing the blot demonstrated no change in the expression of MRP1 (Fig 27B). Fractionation of the CEM-r1 lysate revealed that the majority of MRP4 was found in the membrane fraction (not shown).

To test the functional relationship between MRP4 gene copy and MRP4 expression, CEMss cells were grown stepwise to different levels of PMEA resistance and then samples of DNA, RNA and protein were isolated (Fig 25). The Southern blot revealed negligible changes in MRP4 gene copy for cells grown in up to 2.0 mM PMEA, however, large increases in MRP4 were observed at 10 and 50 mM PMEA (7- and 15-fold, respectively). FISH hybridization analysis of the cells grown in 10 mM and 50 mM PMEA revealed HSRs on chromosome 13 in 92% and 90%, of cells. At 2 mM only one of 50 cells had amplification and this one cell had fewer copies than either the 10 mM or 50 mM cells (not shown). In accord with the increased MRP4 gene copy, MRP4 mRNA strongly increased in cells selected at 10 and 50 mM PMEA. Western blot analysis also indicated a corresponding strong overexpression of immunoreactive MRP4 at 10 mM PMEA and a further increase at 50 mM. It should be emphasized that increased PMEA efflux was observed only when cells overexpressed MRP4 protein and the rate of efflux increased in proportion to the level of MRP4 protein. These studies revealed strong concordance between MRP4 gene copy and expression of MRP4 mRNA and protein.

To confirm that MRP4 confers a dominant phenotype, somatic cell-fusion was used between CEM-r1 and cem.agi.oui.5 [60]. The cem.agi.oui.5 cell line is very sensitive to azaserine induced death due to the absence of hypoxanthine-guanine phosphoribosyl-transferase [60]. The cem.agi.oui.5 cell line is also oubain-resistant [60], but as sensitive to

PMEA as the CEMss cell line (not shown). While the CEM-r1 cells are very PMEA resistant their growth is inhibited by ouabain. Analysis of the fusion cells revealed that all hybrid cells are resistant to PMEA (determined as described in Table 7 legend) and that the range of resistance is from 33.3 to 170 fold, as compared to >250-fold resistance in the CEM-r1 cell line. Immunoblot analysis detected little immunoreactive MRP4 in the cem.agi.oui.5 cell line, but substantial levels of MRP4 in two fusion cell lines (not shown). Southern blot analysis of these cell lines demonstrated increased MRP4 gene copies and FISH analysis of these two fusion cell lines revealed a single copy of chromosome 13 containing a MRP4 HSR in each fusion cell line (not shown).

Because the active forms of these antiretroviral drugs resemble nucleotides the failure to identify a specific transporter linked to nucleotide efflux may be due to the dogma that nucleotides are retained within mammalian cells and not removed by an active transport process. The ABC cassette transporter genes MDR1/Pgp, MRP1, and MRP2 efflux therapeutically important drugs and endogenous substances [64-67], but have not been reported to transport nucleotides. The current studies demonstrate that high levels of the MRP4 gene product are strongly linked to decreased cellular retention and resistance to antiretroviral drugs such as PMEA and AZT. Resistance to PMEA and AZT was associated with MRP4 gene amplification, MRP4 protein overexpression and increased drug efflux, but no change in expression of other ABC transporters (*e.g.* MDR1/Pgp, or MRP1-MRP3). Moreover, without MRP4 overexpression drug efflux was unchanged. PMEA resistance in the absence of MRP4 overexpression is probably due to the previously described change in adenylate kinase [48]. However, AZT is not a substrate for this kinase and there is no evidence for an alteration in thymidine kinase in these cells.

Cells vary in their sensitivity to the cytotoxic effects of AZT [68] and this relates to an unusual feature of AZT, *i.e.*, intracellular accumulation of AZTMP, a finding attributed to the poor substrate activity of AZTMP for thymidylate kinase [69-71]. High levels of cellular AZTMP inhibit the biosynthesis of endogenous deoxynucleoside triphosphates [72] and this activity, is reportedly, related to bone marrow toxicity in patients administered

AZT [72]. AZTMP may also cause cytotoxicity by decreasing glycosphingolipid biosynthesis [73]. The results reported herein demonstrate that intracellular accumulation of AZTMP is diminished in cells that overexpress MRP4 and this facilitates their survival in high concentrations of AZT. Thus, cellular variation in MRP4 amount may cause differential cellular sensitivity to AZT. In support of this, cells previously reported to "excrete" AZTMP into the media (HL60 and K562 [74,75]) have been observed to express high levels of MRP4 (Fig 22).

Cells selected for doxorubicin or cisplatin resistance do not upregulate MRP4 and therefore increased MRP4 is not a general consequence in the development of drug resistance [49]. The present studies suggest that upregulation of MRP4 may specifically occur secondary to the cellular accumulation of cytotoxic monophosphate nucleosides. In support of this is the finding that cells selected for resistance to PMEG (an acyclic nucleoside guanine derivative with reported antineoplastic potential)[76] also express higher levels of MRP4. Clearly, future studies will evaluate the types of monophosphate substrates utilized by MRP4.

These studies demonstrate that increased MRP4 expression directly correlates with decreased intracellular levels of AZTMP and PMEA. An implication of the current findings is that the currently popular, "highly active antiretroviral therapy", (HAART)[77] that utilizes combinations of highly cytotoxic antiretroviral drugs for long time periods may facilitate selection of MRP4 overexpressing cells leading to a failure of antiretroviral drugs to eradicate HIV in these host cells. Furthermore, given the variation in expression of MRP4 in normal tissues [49] and HIVs ability to infect a variety of cell types [78], it is speculated that MRP4 expression forms a basis for sanctuary growth and evolution of drug resistant HIV [79,80] by decreasing the intracellular drug necessary to inhibit viral replication of HIV. While the present studies link MRP4 overexpression with increased efflux of antiretroviral drugs used for HIV, it is not inconceivable that MRP4 expression could impact chemotherapy of herpesvirus, hepatitis B virus and other retroviruses that use drugs of similar structure.

METHODS

Reagents. The following suppliers were used: AZT (Sigma); [3H]-AZT and metabolite standards, AZTMP, AZTDP and AZTTP (Moravek Biochemicals Brea, CA); 3TC (Glaxo Wellcome, Inc. , Research Triangle Park, NC); PMEA, Bis(POM)PMEA (Dr. Norbert Bischofberger, Gilead Sciences, Foster City, CA); MRP1 [64] , MRP2/cMOAT (genBank accession number R02136), MRP3 (genBank accession number T39953), and MRP4 (genBank accession number R35797). All EST cDNAs were sequenced to confirm authenticity as previously reported cDNA clones.

Cell Lines. The human T-lymphoid cell line CEM-ss (National Institute of Health/National Institute of Allergy and Infectious Diseases: AIDS Research and Reference Program, Ogden BioServices, Rockville,MD); CEM-r1, a PMEA-resistant variant of CEMss [48].

MRP4 peptide antiserum. MRP4 was compared with MRP1 (amino acids 1308-1531), MRP2 (cMOAT; amino acids 1315-1545); MRP3, (amino acids 262-485), MRP5 and MRP6 (amino acids, 1178-1401)[49]. Based upon the multisequence MRP alignment and the hydrophobicity/hydrophilicity, the peptide sequence (SGR LKE YDE PYV LLQ NKE SL) was identified, that would be predicted as specific for MRP4 based upon a query of the BLASTP database. This MRP4 peptide was coupled to KLH and used to produce antiserum in rabbits. The antiserum was affinity purified (Research Genetics) using the MRP4 peptide.

MRP4 expression plasmids. A c-terminal fragment of the MRP4 EST containing 171 amino acids was subcloned into the Pinpoint Xa-1 vector (Promega) to generate an in-frame MRP4 expression vector and into Pinpoint Xa-2 to generate an out-of-frame MRP4 expression vector, transformed into bacteria, induced with IPTG and bacterial lysates prepared.

RNase protection analysis. Total cellular RNA was isolated as previously described [49].

An MRP4 RNase protection probe (MRP4-239) was generated using the same primers described by Kool et. al. [49], and MRP4 EST as template, subcloning the 239 bp MRP4 fragment into pCR2.1 (InVitrogen) and verifying the sequence. ^{32}P-labeled MRP4 RNA was transcribed from *SpeI* linearized MRP4-239. RNase protection assays were carried out according to manufacturers instructions (Ambion). Protected probes were visualized by electrophoresis through denaturing gel followed by autoradiography.

Cell fusions. Following the procedure of Wolverton et. al. [86], cells were washed and resuspended in phosphate buffered saline. 2 X 10^6 cells of each cell line to be fused were mixed and pelleted in a 15 ml conical tube at 22°C. The supernatant was discarded such that no more than 50 ul of the supernatant covered the pellet. To the pellet, 0.5 ml of a 50% v/v solution of PEG in RPMI-1640 was added dropwise over a period of 1 min with gentle tapping. Cells were immediately pelleted, resuspended in OHA medium (ouabain (50 nM), hypoxanthine (100 uM), azaserine (600 uM)) plated in a 96-well dish at 2 X 10^4 cells per well and incubated at 37°C in a humidified incubator with 5% CO_2. The cells were nourished with OHA medium at 7 and 14 days post-fusion. Confirmation of cell fusion was determined by both FACS and karyotype analysis.

Several other known inhibitors of ATP-coupled transporters, prostaglandin A_1 (PGA_1), dipyridamole and NBMPR were also examined for their effect on AZTMP efflux in the two cell lines, CEM-SS and CEM-rl. The efflux of AZTMP in CEMrl cells was markedly inhibited by PGA_1 (50 μM) and DPM (10 μM) while no effect was observed in the CEM-SS cells. Also, these compounds did not have any effect on the efflux of AZT itself in either the CEM-SS or the CEM-rl cells.

MATERIALS AND METHODS

Chemicals. PMEA, PMEDAP, PMEG, PMEApp, Bis(POM)PMEA, and monopom-PMEA were kindly provided by Dr. Norbert Bischofberger, Gilead Sciences (Foster City, CA). [2,8-[^{3}H]PMEA (17 Ci/mmol) and [^{3}H]bispom-PMEA (21 Ci/mol) were obtained from Moravek Biochemicals (Brea, CA). The

radioactive compounds were repurified before each experiment. All other nucleoside/nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). AZT was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-AZT and the standards for the metabolites, AZT-MP, AZT-DP and AZT-TP were obtained from Moravek Biochemicals (Brea, CA). 3TC was from Glaxo Wellcome, Inc. (Research Triangle Park, NC).

Cells and virus. The human T lymphoid cell line CEM-SS and HIV-1_{IIIB} were obtained from the National Institutes of Health/ National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Ogden BioServices, Rockville, MD). CEM-rl, a PMEA-resistant variant of CEM-SS, was selected as described (48). In a similar procedure, a PMEG-resistant variant of CEM-SS was also selected. Briefly, CEM-SS cells in culture were exposed to increasing concentrations of PMEG over several months. Of the various lines that grew in the presence of high concentration's of PMEG, one of the cell lines was designated as CEM-RPMEG (100-fold resistance to PMEG, IC_{50} value = 20 mM) and was cultured for at least 10 generations in PMEA-free medium before subsequent studies. CEM-SS, CEM-rl and CEM-RPMEG cells were grown in culture in RPMI-1640 medium (BioWhittaker. Walkersville, MD) supplemented with 10% v:v heat inactivated (56°C, 5 hours) fetal calf serum (HyClone, Logan, UT) and 2 mM L-glutamine (BioWhittaker, Walkersville, MD). The cells were incubated at 37°C in a humidified CO_2 (5%) atmosphere.

All cells were maintained in modified Eagle's medium (BioWhittaker, Walkersville, MD) containing 10% (v/v) heat-inactivated (56°, for 0.5 hr) newborn bovine serum (HyClone, Logan, UT) and 2 mM L-glutamine. HIV-1_{IIIB} was propagated in CEM-SS cells, and cell-free virus stocks were stored in the vapor phase of liquid N_2 cylinders until further use.

Cytotoxicity Assays:

Determination of ED_{50}: The two cell lines, CEM-SS and CEM-rl were infected

with HIV-IMB at a multiplicity of 0.01, and cultured for 6 days in the presence and absence of different concentrations of test drugs. The extent of virus production was monitored by an in-house p24 antigen capture assay (50).

Determination of IC_{50}: All assays were performed in 24-well tissue culture plates (Costar, Cambridge, MA). CEM-SS, CEM-rl or CEM-RPMEG cells were seeded at a density of 2-4 x 10^5 cells/well, in the presence or absence of the test compounds, and were allowed to grow for 48 hr (approximately two doublings) at 37° C in a humidified CO_2-controlled atmosphere. At the end of the incubation, the cell concentrations, sizes, and volumes were counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The data were used to calculate the 50% growth inhibitory concentrations (IC_{50}) of the various test compounds.

RT assays. The antiviral effects of the different drugs were monitored by RT assays according to previously described procedures (20, 21). CEM-SS or CEM-rl cells were infected with an inoculum of HIV-1_{IIIB} standardized to contain 1 RT cpm/cell, and the virus-infected cells were seeded at a concentration of 0.2 x 10^6 cells/ml in medium containing varying concentrations of PMEA (or other test compounds). After 5 days of incubation, the RT activity of the culture supernates was determined by previously described procedures (22). Briefly, the reactions were carried out in a total volume of 50 ul, using 5 μg/ml poly(A)$^+$, 1.6 μg /ml oligo(dT)$_{12-18}$, and 1μCi of [^{3}H]TTP in 50 mM Tris, pH 7.8, 75 mM potassium chloride, 5 mM magnesium chloride, 2 mM dithiothreitol, 0.05% Nonidet P-40. The reaction was initiated by addition of 10ul of virus-containing tissue culture supernatant. After 1 hr of incubation, 10 ul of the reaction mixture were spotted on a Whatman DE-81 filter paper, air dried, and washed four times with 2x standard saline citrate (0.3 M NaCl, 0.03 M sodium citrate). The filter papers were dried, transferred to a plastic bag containing scintillation cocktail, sealed, and counted in an LKB Betaplate reader. The drug concentrations yielding half-maximal RT activity (ED_{50}) was calculated as a measure of the antiviral efficacy of the test

compounds, using the nonlinear curve-fitting software Enzfitter (Elsevier Biosoft, Cambridge, UK).

Uptake and metabolism of [^{3}H]PMEA and [^{3}H]bispom-PMEA. Exponentially growing CEM-SS and CEM-r1 cells were harvested by centrifugation, resuspended at 1 x 10^6 cells/ml in fresh medium in the presence of [^{3}H]PMEA or [^{3}H]bispom-PMEA, and incubated at 37°C. At the indicated time points, aliquots of the cells were removed and centrifuged through Nyosil 50 (W. F. Nye, Inc., New Bedford, MA) at 13,000 *x g* for 60 seconds at 4°C. When bispom-PMEA was investigated, an aliquot of the cell-free medium was also removed and analyzed by HPLC for determination of extracellular metabolites. The cell pellet was extracted with 70% ice-cold methanol and the aqueous phase was collected and analyzed for intracellular tritiated PMEA or tritiated bispom-PMEA and their metabolites by using HPLC analysis, essentially as described previously (23, 24). Peaks were identified by chromatography of authentic standards.

Efflux of [^{3}H]bispom-PMEA and its metabolites. CEM-SS and CEM-r1 cells were pre-incubated at 1 X 10^6 cells/ml with [^{3}H]bispomPMEA (2 μCi/ml) for 15 min, washed with ice-cold medium by centrifugation, resuspended at the same concentration in drug-free medium, and maintained at 37°C. At the indicated times, extracts were prepared from the cells and the medium, and the prodrug and its various nucleoside/nucleotide metabolites were analyzed by HPLC as described previously (24).

Quantitation of AZT and its Metabolites: AZT and its phosphorylated biotransformation products were separated by solid phase extraction (SepPak QMA Cartridges) by using a gradient solvent system that was a slight modification from a previously reported method (51). The cartridges were conditioned with 10% MeOH Ammonium Phosphate Buffer (5 mM, pH 4.0). The sample was loaded onto the column. AZT was eluted with 10% MeOH/Ammonium Phosphate Buffer (5 mm, pH 4.0) and AZTMP was eluted with a 9:1 mixture of 10% MeOH/Ammonium

Phosphate Buffer (5 mm, pH 4.0) and 10% MeOH Ammonium Phosphate Buffer (700 mm, pH 4.6). AZTDP and AZTTP were eluted with 10% MeOH Ammonium Phosphate Buffer (700 mm, pH 4.6). The results are set forth in Table 7 below.

TABLE 7

Phosphorylation Of AZT In CEM-SS And CEM-R1 Cells

Time (Hours)	Intracellular Concentration (μM)				Extracellular Concentration (nM)			
	AZT	AZTMP	AZTDP	AZTTP	AZT	AZTMP	AZTDP	AZTTP
CEM-SS								
4	10	89	0.8	1.3	ND	46	ND	ND
8	8	95	0.7	1.5	ND	151	ND	ND
24	10	50	0.7	1.3	ND	555	ND	ND
CEM-r1								
4	5/1	13.6	0.22	0.36	ND	132	ND	ND
8	5.2	15.9	0.17	0.34	ND	420	ND	ND
24	4.3	12.3	0.21	0.43	ND	1716	ND	ND

Both CEM-SS and CEM-r1 cells were incubated with 10 μM AZT and the phosphorylated biotransformation products of AZT present intracellularly were separated by HPLC and quantitated by liquid scintillation counting.

Uptake of [^{3}H]-AZT and its Metabolites: Exponentially growing CEM-SS and CEM-rl cells were harvested by centrifugation, resuspended at 1 x 10^6 cells/ml in fresh medium in the presence of [^{3}H]-AZT and incubated at 37°C. At various time periods (0, 10, 20, 3 0, 40, 60, 90, 120 seconds) from the start of the incubation, aliquots of the cell suspension were removed and centrifuged through Nyosil 50 (W.F. Nye, Inc.,New Bedford, MA) at 14,000 x g for 60 seconds at 4°C. An aliquot of the top layer (cell free media) was analyzed for the quantitation of AZT and its metabolites. The cell pellet was extracted with 70% methanol/ 15 mM Tris Buffer, pH 7.0(ice-cold) and the aqueous phase was analyzed for AZT and its metabolites.

Efflux of [^{3}H]-AZT and its Metabolites: CEM-SS and CEM-rl cells were pre-incubated at 1 X 10^6 cells/ml with [^{3}H]-AZT (50 μM, 4uCi/ml) for 60 minutes at

37°C. The suspension was centrifuged at 3,300 rpm for 7 minutes. All but 1 ml of the supernatant medium containing the free drug was aspirated. The cells were quickly resuspended in the remaining medium and were centrifuged through Nyosil 50 at 14,000 x g for 60 seconds at 4°C to remove any traces of the labeled drug. The cell pellet was then washed with ice-cold medium and resuspended at the same concentration in drug-free medium and maintained at 37°C. At the indicated time periods, aliquots of the cell suspension were removed and centrifuged through Nyosil 50 at 14,000 x g for 60 seconds at 4°C. An aliquot of the top layer (cell free media) was analyzed for the quantitation of AZT and its metabolites. The cell pellet was extracted with 70% ice-cold methanol and the aqueous phase was analyzed for AZT and its metabolites.

EXAMPLE 2 MRP4 IS A MAMMLIAN NUCLEOSIDE ANALOG EFFLUX PUMP PRESENT IN CEM-r1 CELLS: MOLECULAR AND PROTEIN CHARACTERIZATION OF MRP4.

To identify the efflux pump in the multi-drug resistant cells, described in Example 1, resistant and non-resistant (WT) cellular DNA was analyzed. The results described herein below suggested that MRP4 was associated with the cellular drug resistance. The analysis of genomic DNA from the WT and resistant cells indicated that the resistance phenotype is associated with MRP4 gene amplification.

Southern Hybridization. Restriction digests of the genomic DNA from CEM-SS, CEM-rl and CEM-RPMEG cell lines were probed with ^{32}P-labeled insert from the human cDNA clones for MRP1, MRP2, MRP3 and MRP4. Results are shown in Figure 8. MRP4 was selectively amplified (about 20-fold) in the cell variant selected for resistance to PMEA, while no gene amplification was observed in the cell variant selected for resistance to PMEG. The restriction fragments pattern did not reveal any change in gene structure. In addition, no change was observed in the expression of MRP1, MRP2 and MRP3 in the resistant and the wild type cells.

RNAse Protection Assay. Total RNA from CEM-SS, CEM-rl and CEM-rPMEG

cells was probed with a ^{32}P-labeled RNA probe *in-vitro* transcribed from MRP4-239 and MRP3-200. MRP4 was overexpressed in both the resistant cell variants selected for resistance with PMEA and PMEG. Results are shown in Figure 9.

DNA Sequence Analysis. The human EST database was searched for cDNA clones of MRP homologs. EST clones specific for MRP1 and cMOAT were selected. In addition, two clones (clone # 61401 and clone # 38091 of unknown identity were selected. These clones had an ATP-binding cassette and had 74% and 65% homology, respectively, to the 3' end of CMOAT. Slot blot screening of RNA isolated from the cells using slot blot indicated that clone # 38091 showed a 20-17 fold higher expression of a mRNA compared to the sensitive cell. Recently, Kool et al reported two related clones, clone # 84966 and clone # 38089, identified in the IMAGE Consortium which they designated MRP3 and MRP4 respectively. (49)

The MRP4 EST (GenBank Accession No.: R35798) was obtained from the IMAGE consortium. Sequencing revealed a 2.275 kb insert (see Figure 15) which included an open reading frame representing about 171 amino acids (see Figure 16). An alignment of the MRP4 open reading frame (38091.aa) with the MRP4 EST (labeled MRP4-GenBank) (49) reveals amino acid differences between the two open reading frames and a region of divergence between the two sequences (see Figure 17). A consensus sequence is shown below the alignment of these two sequences.

PCR Assay to Detect Cellular MRP4. Primers were synthesized and used to amplify a 239 bp MRP4 fragment from a first strand cDNA prepared from cellular RNA (See Figure 18). An internal MRP4 PCR control plasmid was prepared from the MRP4 plasmid. This control plasmid has a 65 bp deletion and this reagent and technique is useful to detect small amounts of MRP4 RNA quantitatively in cells in situations when small numbers of cells are available, rendering detection of MRP4 by Western blot not feasible.

The Walker motif is conserved among the ABC cassette transporters and is found as amino acids VGRTGAGKSS (SEQ ID NO.: 16), represented as the following consensus: (G(X4)GKS) (SEQ ID NO.: 13) (FIG 19A-19C). An alignment was performed between the MDR type ABC cassette transporters (Figure 19A) and MRP type (Figure 19B) (49). The motif was identified in both the N-terminal and C-terminal halves of these molecules. The MRP4-EST clone does not contain a Walker A motif (See Figure 19B). Therefore, in order to generate a cDNA clone with additional MRP4 sequence, the Walker motif amino acid sequence was used to generate a degenerate oligonucleotide that in combination with a 3' MRP4-specific oligonucleotide amplify a larger MRP4 cDNA. Poly A mRNA was obtained from PMEA resistant cells and reverse transcribed by priming with oligo-dT. The resulting MRP4 products are of the size predicted by the homology with the other ABC cassette transporters.

Sequence Analysis of the 5' 2.7 kb MRP4 cDNA. The 2.7kb MRP4 cDNA was subcloned into pCR2.1 and sequenced with M13 primers. The sequence analysis reveals that it contains the Walker A motif (Fig 20).

Genomic Analysis. Genomic DNA was isolated from the wild type (CEM-SS) and resistant (CEM-rl) cells according to standard procedures. EcoR I, Hind III and BamH I were used to digest DNA from each cell line. 10 ug of each restriction digest was size fractionated on an 0.8% agarose gel and transferred to a charge modified nylon membrane. Hybridization was performed according to the standard procedures using ^{32}P labeled probes specific for MRP1, MRP2, MRP3 and MRP4. The results indicated that MRP4 was amplified in the resistant cells. (See Figure 8).

Generation of anti-MRP4 antibodies. The MRP4 sequence (38091.aa) was compared with MRP1 (amino acids 1308-1531), MRP2 (cMOAT: amino acids 1315-1545); MRP3 (amino acids 262-485), MRP5 and MRP6 (amino acids 1178-1401). Based upon the hydrophobicity and hydrophilicity profile analysis (see Fig 10) and the multisequence MRP alignment (see Figure 11) two potential peptide

sequences were selected (SEQ ID No.: 17 and SEQ ID No.: 18) because they were predicted to be specific for MRP4 and antigenic. Based upon a query of the BLASTP database (see Figure 12) the following peptide was selected: SGR LKE YDE PYV LLQ NKE SL (SEQ ID No.: 4). The peptide was used to immunize rabbits and was predicted to generate MRP4 specific antibody. The peptide was coupled to KLH prior to inoculation into two rabbits.

Western blot analysis. A C-terminal fragment of MRP4 (71 amino acids representing a Hind III/ Not I fragment was subcloned into the Pinpoint Xa-1 vector to generate an in-frame MRP4 expression vector and Pinpoint Xa-2 to generate an out-of frame MRP4 expression vector. These vectors generate a fusion protein containing MRP4 and a protein that has been tagged with biotin and can therefore be detected by commercial avidin conjugated detection systems using streptavidin alkaline phosphatase detection. These plasmids were transformed into bacteria (see Figure 13). Bacteria containing either the empty vector, in-frame MRP4 or out-of frame MRP4 were subsequently grown overnight diluted and then induced with IPTG. Subsequently, bacterial lysates were prepared and Western blot analysis was performed and the fusion protein detected with streptavidin alkaline phosphatase detection. Fig 13A. Bacterial lysates containing either empty vector, MRP in-frame or MRP4 out of frame were separated on PAGE, transferred to a charged modified membrane and probed with peptide antisera to the epitope in human MRP4 representing amino acids SGR LKE YDE PYV LLQ NKE SL (SEQ ID No.: 4). (Fig 13B).

The bacterial biotinylated MRP4 fusion protein (approximately 31 kD) is recognized by both avidin conjugated alkaline phosphatase and the MRP4 antisera. This antisera was affinity purified and has been used to detect MRP4 overexpression in cells that are resistant to the antiretroviral drugs, AZT PMEA, and PMEG. This antisera is useful in detection of MRP4 in a variety of cell types. Furthermore, the MRP4 biotin fusion construct is valuable for development of a monoclonal antibody to MRP4 after purification of this polypeptide encoding this segment of MRP4.

Western blot analysis (Figure 25C) illustrates specific overexpression of MRP4 compared with MRP1 on same blot after stripping MRP4. Figures 25A and 25B illustrate gene amplification and overexpression of MRP4 respectively.

MATERIALS AND METHODS

cDNA Probes for Southern Hybridizations: Human cDNA clones were obtained from the I.M.A.G.E. consortium and were purchased from Genomic Systems (St. Louis, MO). For MRP4, the insert of a human cDNA clone (no. 38091, Soares Infant Brain INIB- cDNA library), which contained the 3' terminal end of the gene, was sequenced and isolated for use as a probe.

Isolation of Genomic DNA and Southern Blot Analysis: The genomic DNA was isolated from exponentially growing CEM-SS, CEM-rl and CEM-RPMEG cells (1 x 10^8 cells for each cell line) according to the standard procedure [20]. Ten ug of DNA from each cell line was separately digested with EcoRI, Hind III and Bam HI. The restriction digests were electrophoresed on a 0.8 % agarose gel. The DNA fragments were then transferred to a Nylon membrane and UV-cross-linked using a Stratagene UV cross-linker. Blots were pre-hybridized at 42°C for 2 hour in 50% formamide, 5X SSPE buffer (1X = 150 mM NaCl; 10mM NaH4PO, pH 7.0; 1 mM EDTA), 5X Denhardt's, 1% SDS and 100 ug/ml of sheared and sonnicated salmon sperm DNA. The blots were hybridized under the same conditions for 16-20 hours with [α-^{32}P]dCTP-labeled cDNA probe (2.5 x 10^6 cpm/ml; 1 x 10^9 cpm/ug of probe). The blot was then washed twice in 2 X SSC/0.1% SDS for 30 minutes. at 42°C. This was then followed by two consecutive washes in O.1X SSC/0.1% SDS for 30 minutes, the first one at 42°C and the subsequent wash at 55°C.

RNA Isolation: Total RNA was isolated from exponentially growing cells and extracted according to Chomynski and Sacchi.

RNAse Protection Assay: For MRP4, a 239-bp fragment was generated using the primers 5'CCATTGAAGATCTTCCTGG-3'(forward primer) (SEQ ID No.:19) and 5'-GGTGTTCAATCTGTGTGC-3'(reverse primer). (SEQ ID No.: 20). The PCR product was subcloned into PCR2.1 cloning vector (Invitrogen Inc.) resulting in plasmid MRP4-239. The sequences of the inserts were confirmed and inserted in the antisense orientation to the T7 promoter. MRP4-239 was linearized with Spe-I (Promega Corp., Madison, WI) and in-vitro transcription was carried out using T7 RNA Polymerase (Ambion, Inc.) and α^{32}P-CTP to generate an MRP4 riboprobe. RNAse Protection Assay was carried out using the procedure recommended by the manufacturer of the kit (Ambion Inc.). The protected fragments were visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. The amount of MRP4 RNA was calculated using a phosphorimager.

Cell Fusion: Cell fusions were carried out according to the procedure of Wolverton et. al. (60). All steps were carried out at room temperature unless mentioned otherwise. Cells were washed and resuspended in phosphate buffered saline. For the fusion of any two cell variants, 2 x 10^6 cells of each cell line were mixed and pelleted in a 15-ml conical tube. The supernatant was discarded such that no more than 50 ul of the supernatant covered the pellet. To the pellet, 0.5 ml of a 50% v/v solution of PEG in RPMI-1640 was added dropwise over a period of 1 minute with gentle tapping of the tube. Cells were immediately pelleted, carefully resuspended in OHA medium and plated in 96-well dishes at 2 x 10^4 cells per well. The plates were incubated at 37°C in a humidified incubator jacket with 5% $C0_2$. The cells were nourished with OHA medium at 7 and 14 days post-fusion.

EXAMPLE 3 REGULATION OF MRP4 EXPRESSION IN TUMOR CELLS.

Although MRP4 appears to be expressed in a limited number of normal tissues (49) studies suggest that it is widely expressed in a variety of tumor cells (see Figure 22) from a variety of lineages representing reproductive epithelia (Hela), hematopoietic

cells (HL60, molt4), lung (A459) and colon carcinoma (SW480, also CaCo-2) (see Figure 22). Because of the high MRP4 expression in K562 and SW480, both of which are p53 null, it was determined whether p53 might play a role in the regulation of MRP4. To accomplish this a K562 cell line was developed that expressed a temperature sensitive p53 allele, such that at 32°C p53 was in the wildtype conformation and at 37°C p53 was in the mutant, non-functional conformation (see Figure 23). These studies revealed that after either 24 or 48 h at 32°C the level of MRP4 protein was substantially reduced. In contrast, the temperature shift only had a minimal effect on MRP4 expression in the control cells (labeled vc). These studies reveal that the MRP4 gene is repressed by the wildtype p53 and imply that p53 mutation will lead to an upregulation in MRP4 expression thus facilitating resistance to MRP4 substrates.

EXAMPLE 4 PMEA TRANSPORT IN A INTESTINAL CELL LINE

Western blot analysis revealed that MRP4 is expressed in LLCPK1 cells (a kidney epithelial cell line) and the CaCo2 cells (an intestinal cell line). Both of these cells polarize in culture to reveal whether a transporter segregates to either the basal or apical membrane.

A transwell culture which is permissive for cellular polarization may be used to measure the compartment to compartment flux of putative substrates. Evidence obtained by confocal microscopy revealed that the CaCo-2 cells express MRP4 on the basal lateral membrane and not the apical membrane. Furthermore it has been shown in transwell culture of CaCo-2 that PMEA is more rapidly transported from the apical to the basal side, thus indicating basolateral expressed MRP4 is functional too (see Figure 24). In addition, it has been determined that prostaglandin A specifically inhibits the apical to basal transport, but has no effect on basal to apical flux. This analysis reveals that specific inhibitors of PMEA mediated transport can be identified in this transwell culture system. In addition, it is possible that expression of MPR4 depending if it is basal or apical (and this may vary by organ

cultured) may determine if PMEA or PMEA-like drugs are absorbed.

EXAMPLE 5 DIAGNOSTIC ASSAY FOR MRP4 LEVELS

To determine the amount of MRP4 contained in a sample several diagnostic techniques are used. The sample is prepared in a buffer that stabilizes protein and RNA. Determination of the amount of the protein is performed by immunochemical analysis using either Western blot or immunohistochemistry. A positive control sample from a cell expressing immunoreactive MRP4 may be included for analysis. MRP4 is detected using standard immunochemical methods.

In order to evaluate the amount of MRP4 in an unknown sample the amount of the positive control may be varied and its immunochemical signal be determined. The signal strength can vary as a function of the amount of the immunoreactive MRP4 and is used to construct a standard curve. From the standard curve an estimate of the amount of MRP4 in the unknown sample is performed.

For RNA quantification of MRP4 a cDNA amplification procedure is used based upon reverse-transcriptase generation of a MRP4 cDNA. The kit provides the oligonucleotide primers spanning the region of MRP4 to be amplified and a MRP4 internal control plasmid. In addition, a complementary MRP4 cRNA is included. The MRP4 internal control plasmid contains a small deletion that upon amplification generates a product smaller than that generated from the reverse-transcribed MRP4 cDNA. Thus, based on size, the product of the internal control is readily distinguished from the authentic MRP4 obtained from the patient sample. The ratio of the signal obtained from the patient to the internal control allows estimation of the amount of MRP4 mRNA contained in a sample.

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This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

WHAT IS CLAIMED IS:

1. An isolated multi-drug resistance protein 4 (MRP4) nucleic acid having the following characteristics:

(a) encoding a MRP4 protein; and

(b) the ability to hybridize under standard hybridization conditions to the sequence shown in SEQ. ID.No.:1 or a portion thereof.

2. The nucleic acid of claim 1 comprising SEQ.ID.No.:1 or a portion thereof.

3. The nucleic acid of claim 1, wherein the nucleic acid is selected from the group consisting of DNA, RNA and cDNA.

4. A vector comprising the nucleic acid of claim 1.

5. The vector of claim 4, wherein the vector comprises viral or plasmid DNA.

6. An expression vector comprising the nucleic acid of claim 1 and regulatory elements.

7. A host vector system which comprises the expression vector of claim 6 in a suitable host.

8. A vector, comprising cDNA encoding MRP4 (ATCC Accession No.:_____).

9. The host vector system of claim 7, wherein the suitable host is selected from the group consisting of a bacterial cell, a eukaryotic cell, a mammalian cell and an insect cell.

10. An isolated MRP4 protein or a portion thereof.

11. The protein of claim 10, wherein MRP4 has substantially the same amino acid sequence as shown in SEQ.ID.No.: 2.

12. A nucleic acid probe capable of specifically hybridizing with the nucleic acid of claim 1.

13. The nucleic acid probe of claim 12, wherein the probe is capable of specifically hybridizing with a nucleic acid selected from the group consisting of SEQ.ID.No:5, SEQ.ID.No:6, SEQ.ID.No:7, and SEQ.ID.No:8.

14. An antibody capable of specifically binding to the protein of claim 10 without substantially cross-reacting with non MRP4 proteins or homologs thereof under conditions permissive to antibody binding.

15. A cell capable of producing the antibody of claim 14.

16. A method of identifying the protein of claim 10 in a sample comprising:

a) contacting the sample with the antibody of claim 14 under conditions permissive to the formation of a complex between the antibody and the protein;

b) determining the amount of complex formed; and

c) comparing the amount of complex formed in step (b) with the amount of complex formed in the absence of the antibody, the presence of an increased amount of complex formed in the presence of the antibody indicating identification of the protein in the sample.

17. A method for identifying a nucleic acid in a sample which encodes MRP4 protein which comprises:

(a) contacting the sample with a nucleic acid probe of claim 12 under conditions permissive to the formation of a complex between the nucleic acid probe and the nucleic acid encoding the MRP4 protein in

the sample;

(b) determining the amount of complex formed in step (a); and

(c) comparing the amount of complex determined in step (b) with the amount of complex formed using an arbitrary sequence, a greater amount of complex formed with the MRP4-specific probe indicating the presence of a nucleic acid encoding a MRP4 protein in the sample.

18. The method of claim 17, step (a) further comprising amplifying the nucleic acid molecule encoding the MRP4 protein under conditions suitable for polymerase chain reaction.

19. The method of claim 17, wherein the amplified nucleic acid molecule encoding MRP4 is detected by size fractionation.

20. The method of claim 17, wherein the probe is labeled with a detectable marker.

21. The method of claim 20, wherein the detectable marker is a radiolabeled molecule, a fluorescent molecule, an enzyme, a ligand, or a magnetic bead.

22. An isolated nucleic acid, previously unknown, identified by the method of claim 17.

23. A method for identifying a compound that modulates expression of MRP4 comprising:

(a) contacting a sample which expresses MRP4 with the compound;

(b) determining the amount of expression of MRP4 protein in the sample; and

(c) comparing the amount of MRP4 protein expression determined in step (b) with the amount determined in the absence of the compound.

24. The method of claim 23, wherein the sample is selected from the group consisting of cell lysate, a cell-free translation expression system, an isolated cell and a cultured host cell.

25. A compound, previously unknown, identified by the method of claim 23.

26. A method for identifying a compound capable of modulating MRP4 protein activity comprising:

(a) contacting a sample which expresses MRP4 protein with the compound;

(b) determining the amount of MRP4 protein activity in the sample; and

(c) comparing the amount of MRP4 protein activity determined in step (b) with the amount determined in the absence of the compound, an increase or decrease in activity indicating the presence of a compound capable of modulating the MRP4 protein activity.

27. The method of claim 26, step (a) comprising first introducing the nucleic acid encoding a MRP4 protein into an expression system and causing the expression system to express the nucleic acid under conditions whereby a MRP4 protein is produced.

28. The method of claim 26, wherein step (b) comprises measuring the efflux of an antimicrobial agent from a cell in the presence of the compound.

29. The method of claim 28, wherein the agent is selected from the group consisting of nucleoside inhibitors and protease inhibitors.

30. The method of claim 29, wherein the agent is AZT.

31. The method of claim 26, wherein the sample is selected from the group

consisting of cell lysate, a cell-free translation expression system, an isolated cell and a cultured host cell.

32. A compound, previously unknown, identified by the method of claim 26.

33. The method of claim 24 or 26, wherein the compound is a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule.

34. The method of claim 24 or 26, wherein the compound is bound to a solid support.

35. A method of modulating MRP4 protein activity in a sample, comprising contacting the sample with the compound of claim 32.

36. A pharmaceutical composition which comprises the compound of claim 32 and a pharmaceutically acceptable carrier.

37. The pharmaceutical composition of claim 36, wherein the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

38. A method for treating a condition in a subject which comprises administering to the subject an amount of the pharmaceutical composition of claim 36, effective to treat the condition in the subject.

39. The method of claim 38, wherein the condition is selected from the group consisting of an infectious, immunodeficiency, neurological, renal. pulmonary, hepatic, cardiovascular, neoplastic and malignant condition.

40. The method of claim 39, wherein the condition is a result of virus, bacterial, or yeast infection.

41. A method for identifying subjects at risk for resistance to anti-microbial agent therapy comprising:

(a) identifying by the method of claims 24 or 26, the presence of MRP4 in a sample from the subject;

(b) measuring the amount of MRP4 present in the sample from the subject;

(c) comparing the amount of MRP4 present in a control sample having an amount of MRP4 which does not indicate resistance to drug therapy, an elevated amount of MRP4 present in the sample from the subject indicating increased risk for resistance to drug therapy in the subject.

42. A method of identifying an anti-microbial agent which is refractive to MRP4 efflux activity comprising:

(a) Contacting a cell expressing MRP4 with the agent;

(b) measuring the amount of agent in the cell;

(c) Incubating the cell with the agent;

(d) Comparing the amount of agent in the cell before and after the incubation of step (c) no substantial decrease in the amount of the agent in the cell after the incubation of step (c) indicating a agent which is refractive to MRP4 efflux activity.

43. The method of claim 42, step (a) further comprising labeling the agent with a detectable marker.

44. The method of claim 43, wherein the detectable marker is a radiolabeled molecule, a fluorescent molecule, an enzyme, a ligand, or a magnetic bead.

45. The agent identified by the method of claim 42.

46. A pharmaceutical composition comprising the agent of claim 42 and a pharmaceutically acceptable carrier.

47. A transgenic non-human animal whose somatic and germ cells contain and express a gene encoding MRP4 protein the gene having been introduced into the animal or an ancestor of the animal at an embryonic stage and wherein the gene may be operably linked to an inducible promoter element.

48. The animal of claim 47, wherein the animal is a mouse.

49. The transgenic animal of claim 47 wherein the gene encoding MRP4 is overexpressed.

50. A transgenic animal of claim 47 wherein the transgenic animal is a knockout, comprising a genetic mutation which substantially reduces expression of MRP protein under normal conditions.

51. A cell isolated from the transgenic animal of claim 47.

52. A method of identifying a antimicrobial agent which is refractive to MRP4 efflux comprising the steps of:

(a) Contacting the cell of claim 51 with the agent;

(b) Measuring the amount of agent in the cell;

(c) Incubating the cell with the agent;

(d) Comparing the amount of agent in the cell before and after the incubation of step (c) no substantial decrease in the amount of the agent in the cell after the incubation of step (c) indicating a agent which is refractive to MRP4 efflux.

53. The method of claim 52 wherein the transgenic animal overexpresses MRP4 protein.

54. A diagnostic kit to facilitate diagnosis and treatment of a multidrug resistance-related condition comprising: (a) the antibody of claim 14.

55. The diagnostic kit of claim 54, further comprising: (b) reagents which facilitate binding of the antibody to MRP4 or a portion or subunit thereof.

56. The diagnostic kit of claim 54, wherein the antibody is labeled.

57. A diagnostic kit to facilitate diagnosis and treatment of a multidrug resistance-related condition comprising: (a) the nucleic acid probe of claim 12.

58. The diagnostic kit of claim 57, further comprising: (b) reagents which facilitate hybridization of the nucleic acid probe with MRP4 in a sample.

59. The diagnostic kit of claim 57, further comprising: (b) reagents which facilitate polymerase chain reaction of MRP4.

60. The diagnostic kit of claim 57, wherein the nucleic acid probe is labeled.

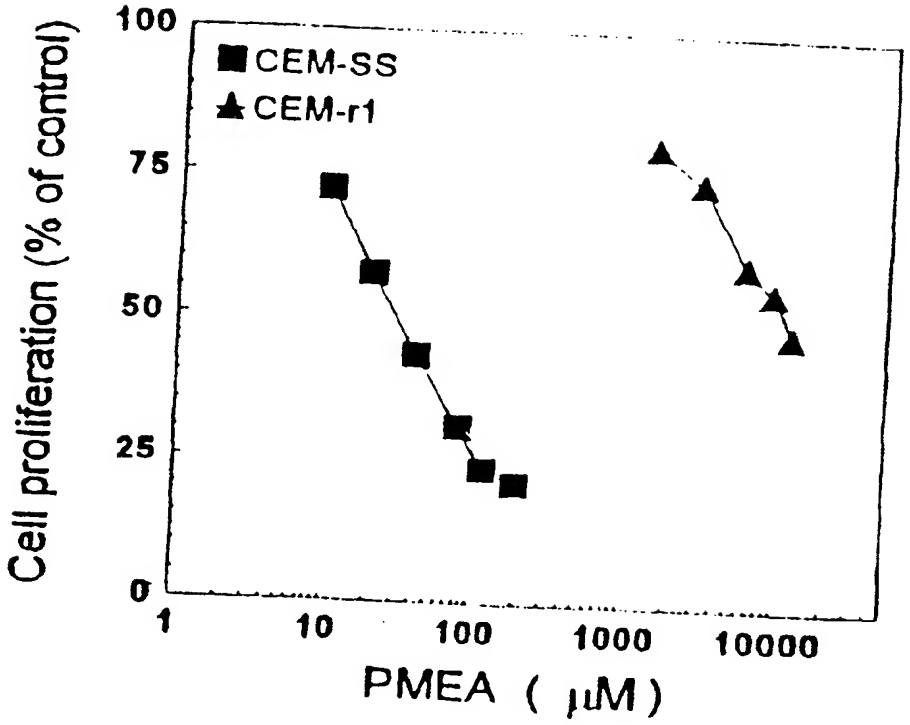

100
75
50
25
0
Cell proliferation (% of control)
CEM-SS
CEM-r1
1
10
100
1000
10000
PMEA (μM)

FIGURE 1

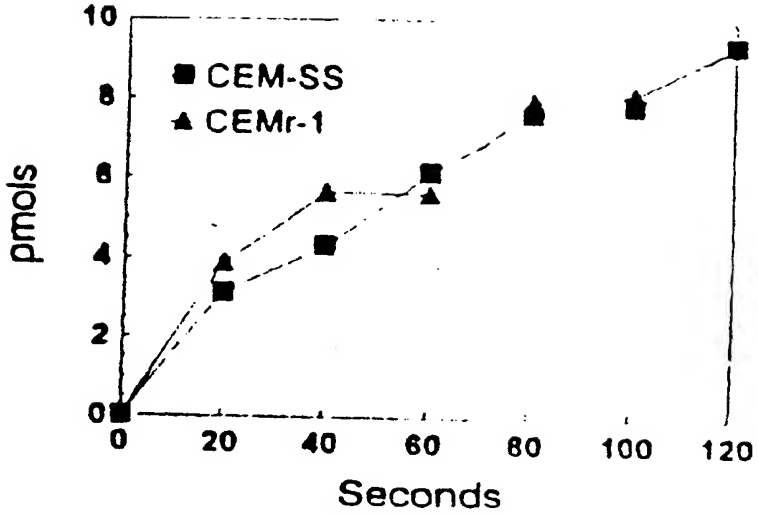


FIGURE 2

FIGURE 3A

FIGURE 3B

FIGURE 3C

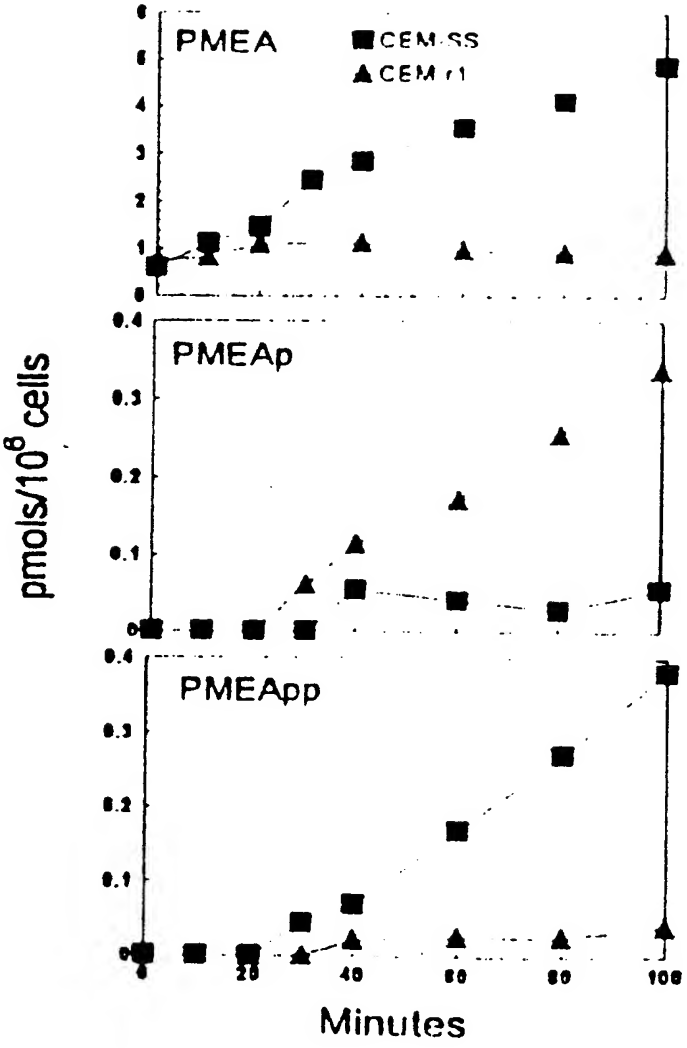
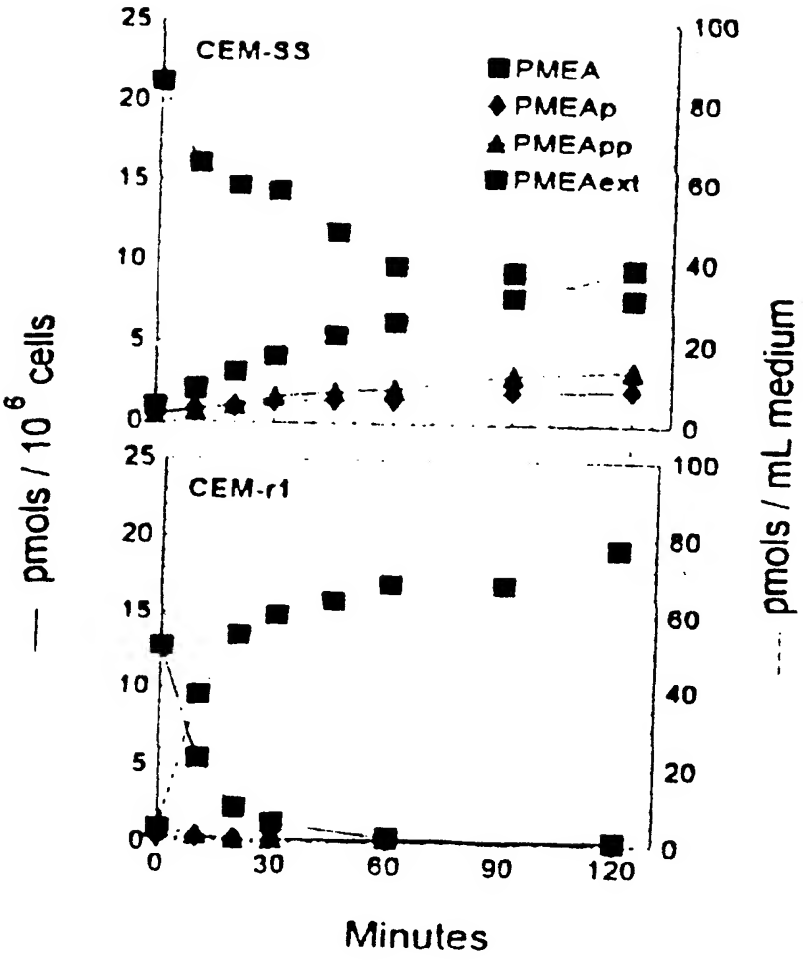


FIGURE 4A

FIGURE 4B



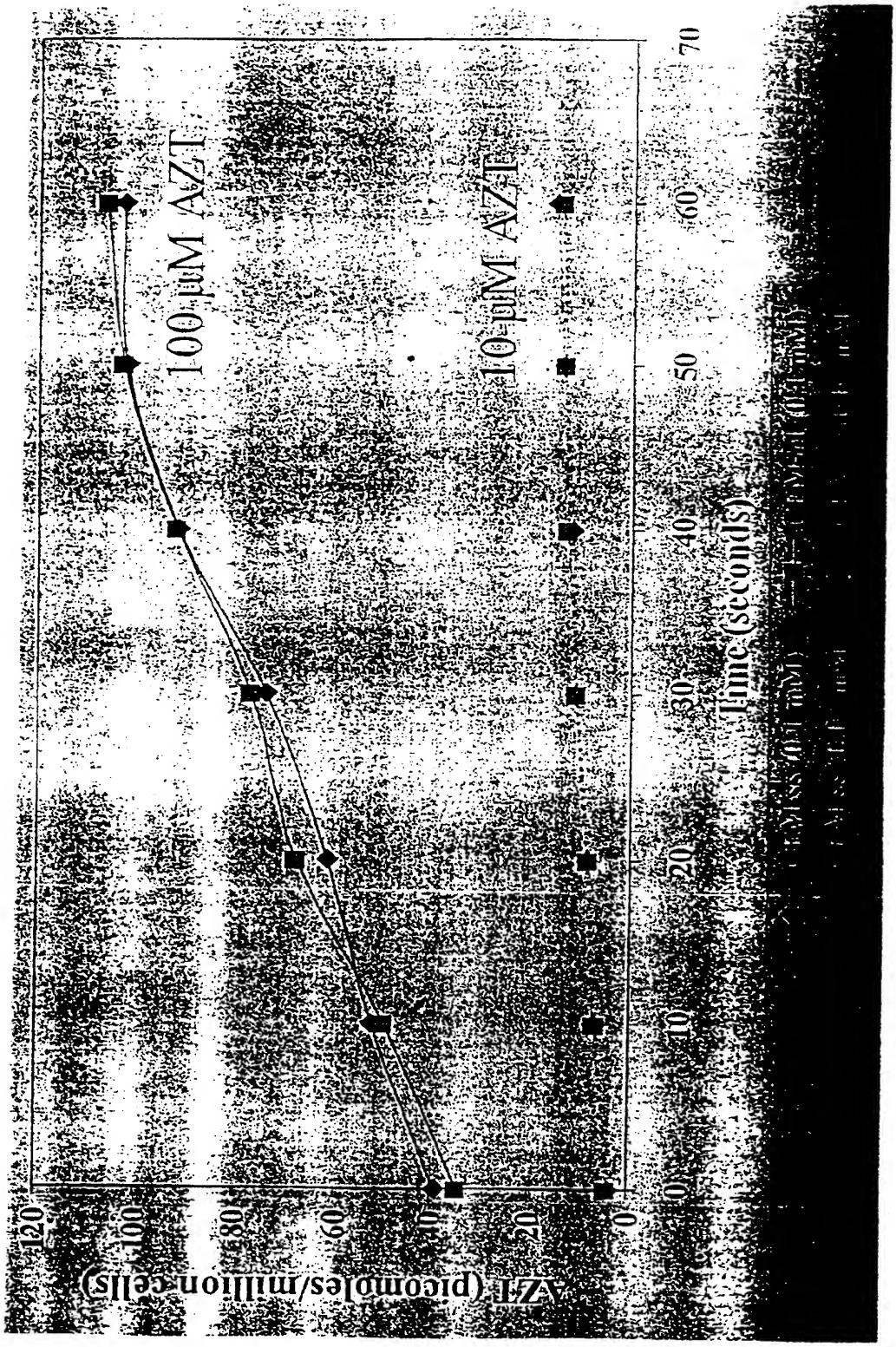

Comparison of AZT Uptake in CEM-SS and CEM-r1
AZT (picomoles/million cells)
Time (seconds)
100 µM AZT
10 µM AZT
0
20
40
60
80
100
120
0
10
20
30
40
50
60
70

FIGURE 5

Fig.2 **AZTMP Efflux in Resistant Variants**

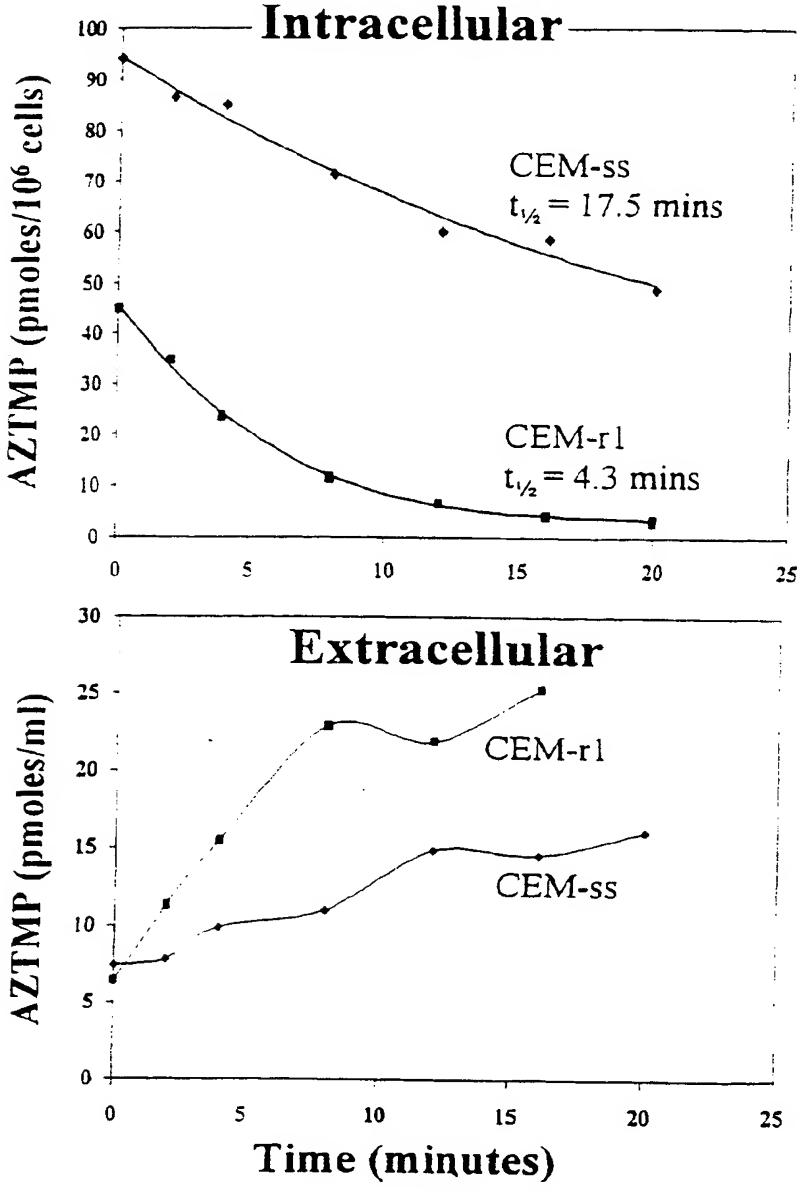


FIGURE 6A

FIGURE 6B

FIGURE 6

FIGURE 7A

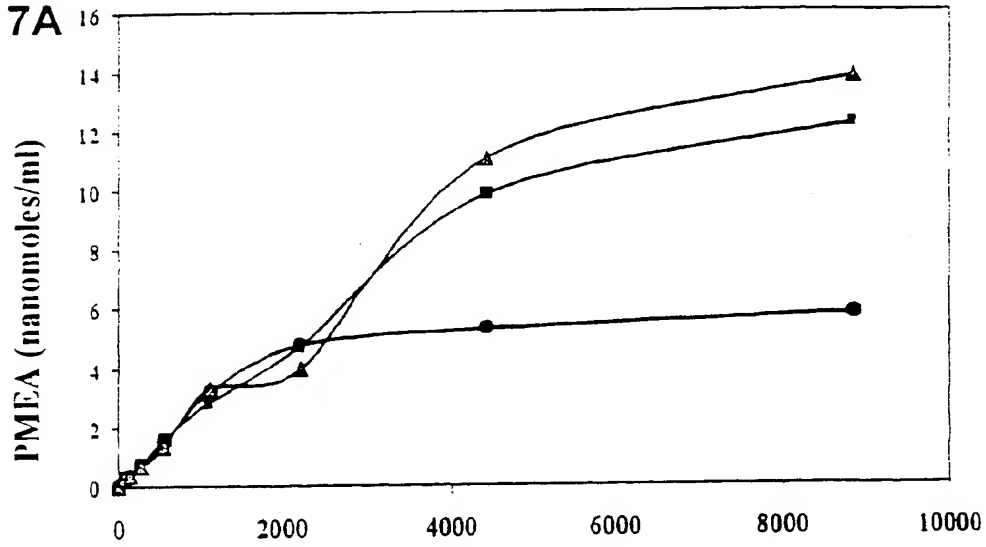


FIGURE 7B

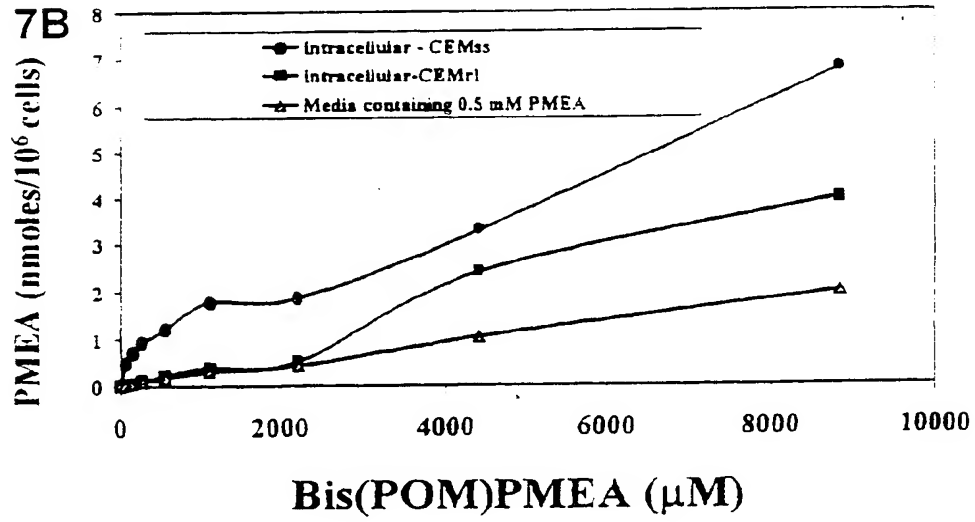


FIGURE 7

Selective Amplification of *MRP4* in CEM-r1

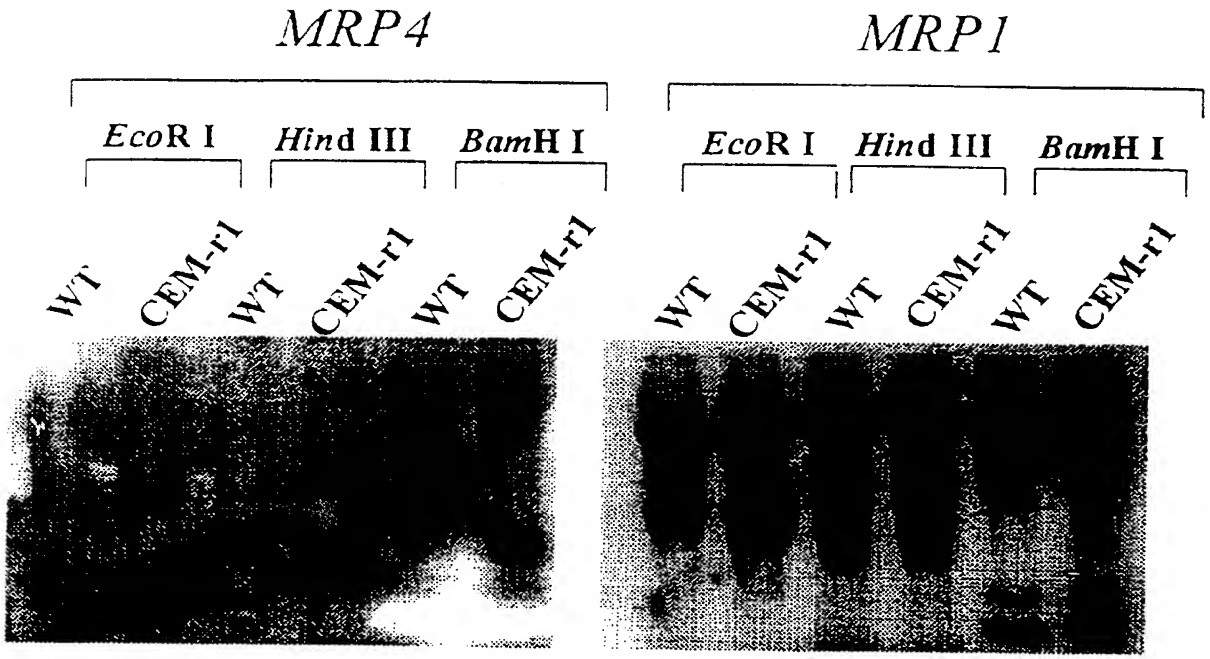


FIGURE 8A

FIGURE 8B

FIGURE 8

RNase Protection Analysis of *MRP4* Expression in WT and CEM-r1 Cells

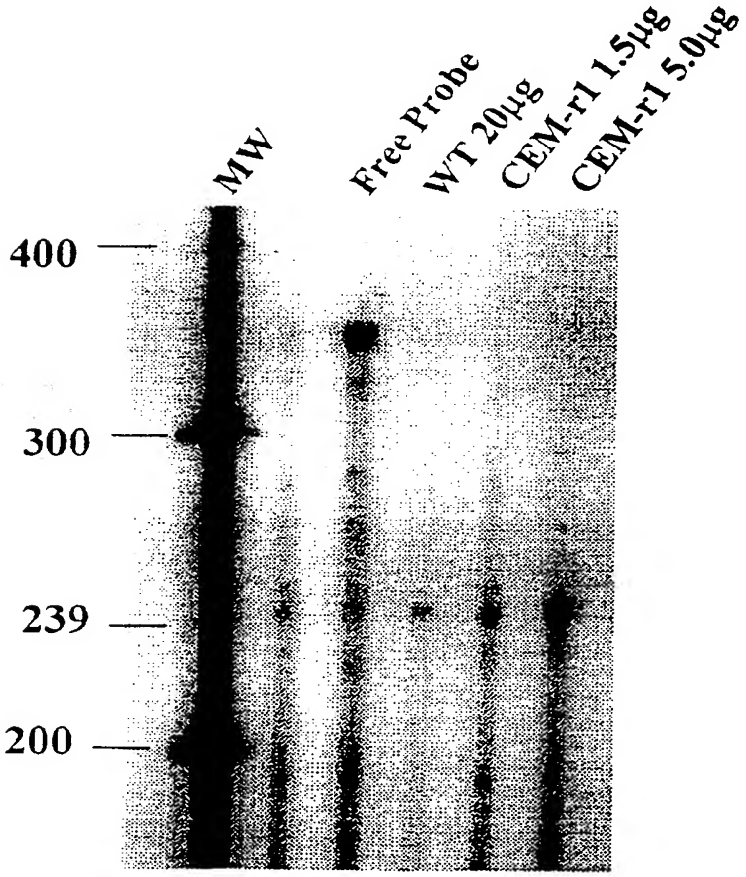


FIGURE 9

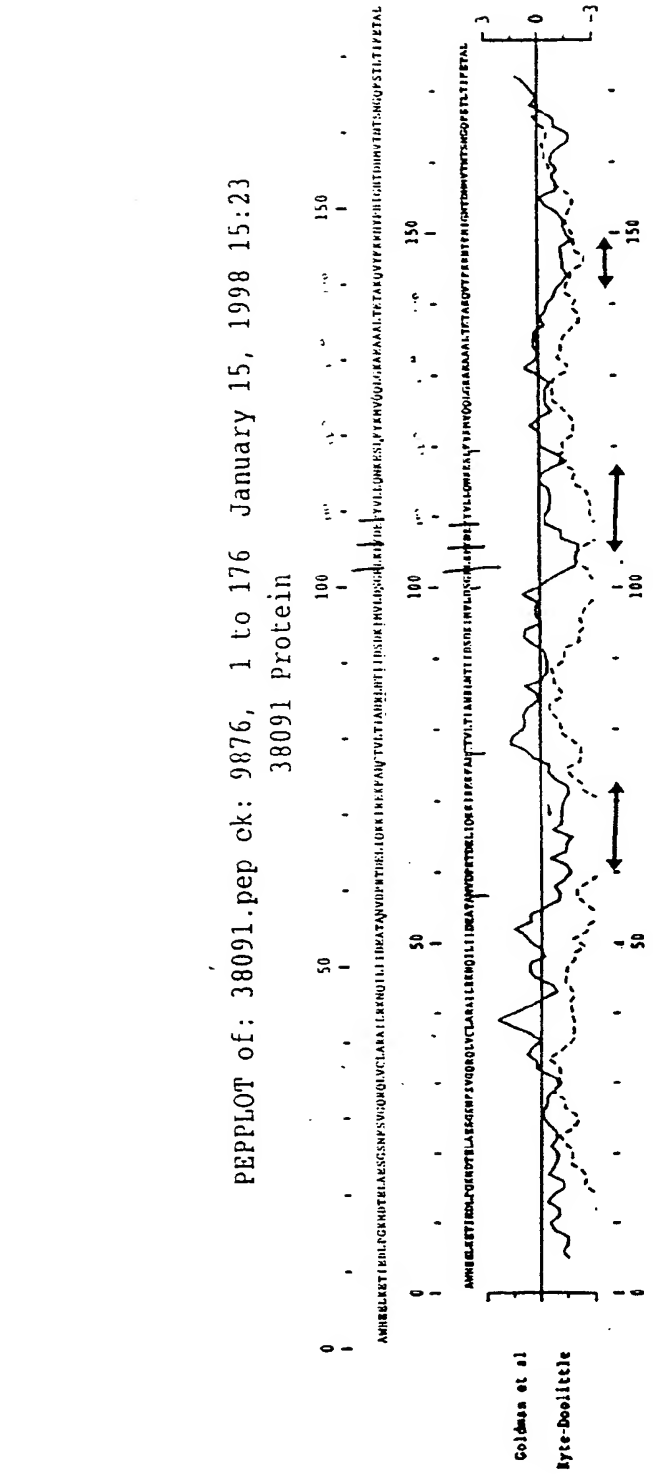

PEPPLOT of: 38091.pep ck: 9876, 1 to 176 January 15, 1998 15:23
38091 Protein
HPhobic
HPhilic
Kyte-Doolittle
Goldman et al

FIGURE 10

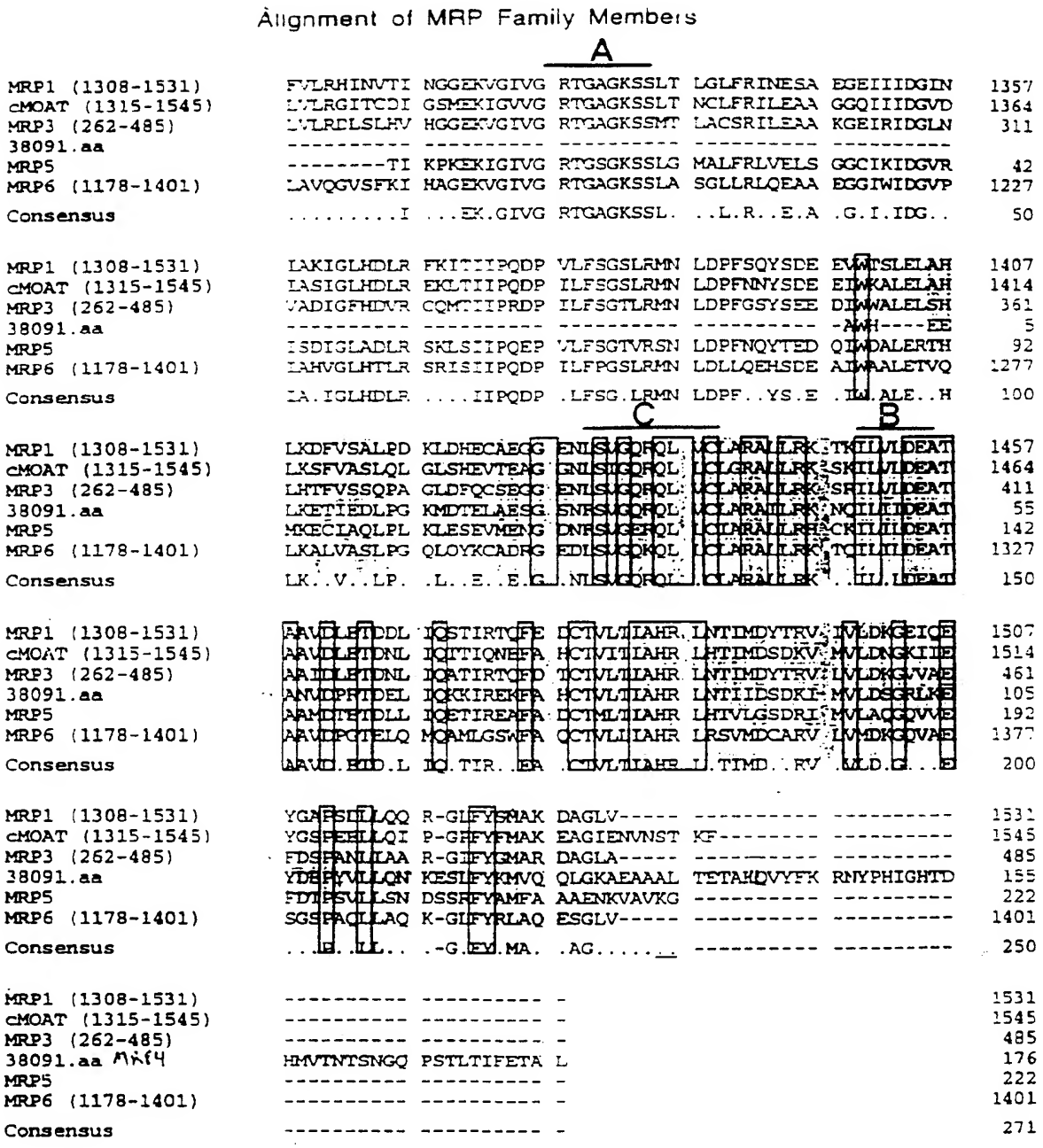

Alignment of MRP Family Members
A
C
B
MRP1 (1308-1531)
cMOAT (1315-1545)
MRP3 (262-485)
38091.aa
MRP5
MRP6 (1178-1401)
Consensus

FIGURE 11

NCBI BLAST Search Results Entrez ?

Reference: Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Query= MRP4 Polypeptide Choice 1 (aa100-119)
(20 letters)

Database: Non-redundant GenBank CDS translations+PDB+SwissProt+SPupdate+PIR
285,190 sequences; 85,415,576 total letters.

Searching.....done

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
gi|2439972	(U83660) multidrug resistance-associ...	102	3.9e-06	1
pir||S64800	probable membrane protein YLL048c - ...	66	0.22	1
sp|P36171|YKR4_YEAST	PROBABLE ATP-DEPENDENT TRANSPORTER Y...	61	0.68	1
pir||A32126	155K membrane glycoprotein - goat (f...	58	0.94	1

gi|2439972 (U83660) multidrug resistance-associated protein homolog [Homo sapiens]
Length = 162

Score = 102 (45.2 bits), Expect = 3.9e-06, P = 3.9e-06
Identities = 20/20 (100%), Positives = 20/20 (100%)

```
Query:     1 SGRLKEYDEPYVLLQNKESL 20
             SGRLKEYDEPYVLLQNKESL
Sbjct:    95 SGRLKEYDEPYVLLQNKESL 114
```

pir|S64800 probable membrane protein YLL048c - yeast (Saccharomyces cerevisiae) gnl|PID|e245758 (Z73153) ORF YLL048c [Saccharomyces cerevisiae]
Length = 1661

Score = 66 (29.2 bits), Expect = 0.24, P = 0.22
Identities = 12/19 (63%), Positives = 15/19 (78%)

```
Query:     1 SGRLKEYDEPYVLLQNKES 19
             +G +KEYD PY LL NK+S
Sbjct:  1611 AGEVKEYDHPYSLLLNKDS 1629
```

sp|P36171|YKR4_YEAST PROBABLE ATP-DEPENDENT TRANSPORTER YKR104W
pir||S30183 probable purine nucleotide-binding protein YKR104w - yeast (Saccharomyces cerevisiae) gi|486611 (Z28329) ORF YKR104w [Saccharomyces cerevisiae]
Length = 306

Score = 61 (27.0 bits), Expect = 1 1, P = 0.68
Identities = 9/19 (47%), Positives = 15/19 (78%)

```
Query:     2 GRLKEYDEPYVLLQNKESL 20
             G+KEYD PY L+ ++ ++
Sbjct:   262 GRVKEYDHPYTLISDRNTI 280
```

pir||A32126 155K membrane glycoprotein - goat (fragments)
Length = 104

Score = 58 (25.7 bits), Expect = 2 8, P = 0 94
Identities = 10/15 (66%), Positives = 14/15 (93%)

```
Query:     3 RLKEYDEPYVLLQNK 17
             +L+EYD+P+VLL NK
Sbjct:    12 KLEEYDKPHVLLQNK 26
```

Parameters:

V=100
B=50
H=0

Lambda K H
0.307 0.133 0.355

Cutoff to enter 2nd pass: >= 36 (0.0 bits)

E	S	T1	T2	X1	X2	W	Gap
10.0	58	11	11	-16	-23	40	50

Database: Non-redundant GenBank CDS translations+PDB+SwissProt+SPupdate+PIR
Posted date: Jan 21, 1998 7:53 AM
# of letters in database: 85,415,576
# of sequences in database: 285,190

Number of Hits to DB: 1st pass: 1105475, 2nd pass: 15562
Number of Sequences: 1st pass: 285190, 2nd pass: 430
Number of extensions: 1st pass: 27037, 2nd pass: 11491
Number of successful extensions: 1st pass: 430, 2nd pass: 857
Number of sequences better than 10: 4

FIGURE 12

FIGURE 13A

Streptavidin Alkaline Phosphatase

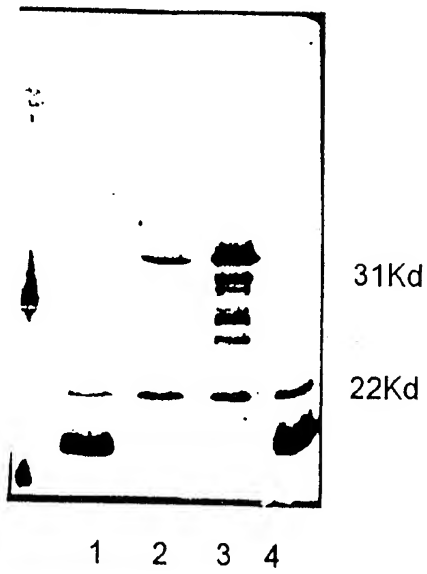


FIGURE 13B

Rabbit anti-MRP4

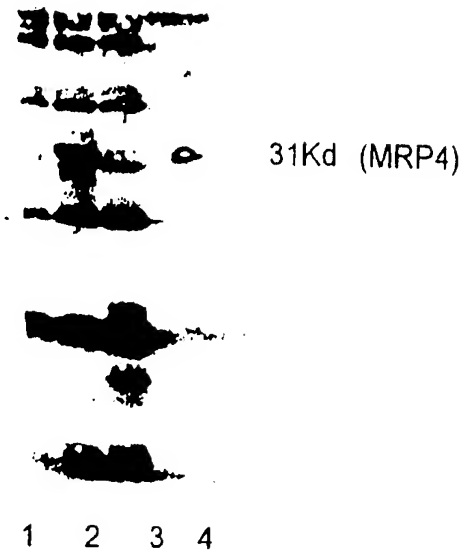


FIGURE 13

MRP4 is Specifically Overexpressed in 50^{MT} Cells

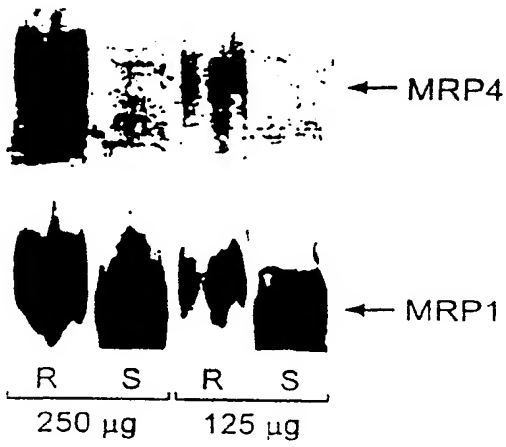


FIGURE 14

```
ACTTAAAGAA ACCATTGAAG ATCTTCCTGG TAAAATGGAT ACTGAATTAG CAGAATCAGG      60
ATCCAATTTT AGTGTTGGAC AAAGACAACT GGTGTGCCTT GCCAGGGCAA TTCTCAGGAA     120
AAATCAGATA TTGATTATTG ATGAAGCGAC GGCAAATGTG GATCCAAGAA CTGATGAGTT     180
AATACAAAAA AAAATCCGGG AGAAATTTGC CCACTGCACC GTGCTAACCA TTGCACACAG     240
ATTGAACACC ATTATTGACA GCGACAAGAT AATGGTTTTA GATTCAGGAA GACTGAAAGA     300
ATATGATGAG CCGTATGTTT TGCTGCAAAA TAAAGAGAGC CTATTTTACA AGATGGTGCA     360
ACAACTGGGC AAGGCAGAAG CCGCTGCCCT CACTGAAACA GCAAAACAGG TATACTTCAA     420
AAGAAATTAT CCACATATTG GTCACACTGA CCACATGGTT ACAAACACTT CCAATGGACA     480
GCCCTCGACC TTAACTATTT TCGAGACAGC ACTGTGAATC CAACCAAAAT GTCAAGTCCG     540
TTCCGAAGGC ATTTTCCACT AGTTTTTGGA CTATGTAAAC CACATTGTAC TTTTTTTTAC     600
TTTGGCAACA AATATTTATA CATACAAGAT GCTAGTTCAT TTGAATATTT CTCCCAACTT     660
ATCCAAGGAT CTCCAGCTCT AACAAAATGG TTTATTTTTA TTTAAATGTC AATAGTTGTT     720
TTTTAAAATC CAAATCAGAG GTGCAGGCCA CCAGTTAAAT GCCGTCTATC AGGTTTTGTG     780
CCTTAAGAGA CTACAGAGTC AAAGCTCATT TTTAAAGGAG TAGGACAAAG TTGTCACAGG     840
TTTTTGTTGT TGTTTTTATT GCCCCCAAAA TTACATGTTA ATTTCCATTT ATATCAGGGA     900
TTCTATTTAC TTGAAGACTG TGAAGTTGCC ATTTTGTCTC ATTGTTTTCT TTGACATAAC     960
TAGGATCCAT TATTTCCCCT GAAGGCTTCT TGTTAGAAAA TAGTACAGTT ACAACCAATA    1020
GGAACAACAA AAAGGAAAAA GTTTGTGACA TTGTAGTAGG GAGTGTGTAC CCCTTACTCC    1080
CCATCAAAAA AAAAATGGAT ACATGGTTAA AGGATAGAAG GGCAATATTT TATCATATGT    1140
TCTAAAAGAG AAGGAAGAGA AAATACTACT TTCTCAAAAT GGAAGCCCTT AAAGGTGCTT    1200
TGATACTGAA AGACACAAAT GTGACCGTCC ATCCTCCTTT AGAGTTGCAT GACTTGGACA    1260
CGGTAACTGT TGCAGTTTTA GACTCAGCAT TGTGACACTT CCCAAGAAGG CCAAACCTCT    1320
AACCGACATT CCTGAAATAC GTGGCATTAT TCTTTTTTGG ATTTCTCATT TATGGAAGGC    1380
TAACCCTCTG TTGACCGTAA GCCTTTTGGT TTGGGCTGTA TTGAAATCCT TTCTAAATTG    1440
CATGAATAGG CTCTGCTAAC GTGATGAGAC AAACTGAAAA TTATTGCAAG CATTGACTAT    1500
AATTATGCAG TACGTTCTCA GGATGCATCC AGGGGTTCAT TTTCATGAGC CTGTCCAGGT    1560
TAGTTTACTC CTGACCACTA ATAGCATTGT CATTTGGGCT TTCTGTTGAA TGAATCAACA    1620
AACCACAATA CTTCCTGGGA CCTTTTGTAC TTTATTTGAA NTATGAGTCT TTAATTTTTC    1680
CCTGATGATG GTGGCTGTAA TATGTTGAGT TCAGTTTACT AAAGGTTTTA CTATTATGGT    1740
TTGAAGTGGA GTCTCATGAC CTCTCAGAAT AAGGTGTCAC CTCCCTGAAA TTGCATATAT    1800
GTATATAGAC ATGCACACGT GTGCATTTGT TTGTATACAT ATATTTGTCC TTCGTATAGC    1860
AAGTTTTTTG CTCATCAGCA GAGAGCAACA GATGTTTTAT TGAGTGAAGC CTTAAAAAGC    1920
ACACACCACA CACAGCTAAC TGCCAAAATA CATTGACCGT AGTAGCTGTT CAACTCCTAG    1980
TACTTAGAAA TACACGTATG GTTAATGTTC AGTCCAACAA ACCACACACA GTAAATGTTT    2040
ATTAATAGTC ATGGTTCGTA TTTTAGGTGA CTGAAATTGC AACAGTGATC ATAATGAGGT    2100
TTGTTAAAAC GATAGCTATA TTCAAAATGT CTATATGTTT ATTTGGACTT TTGAGGTTAA    2160
AGACAGTCAT ATAAACGTCC TGTTTCTGTT TTAATGTTAT CATAGAATTT TTTAATGAAA    2220
CTAAATTCAA TTGAAATAAA TGATAGTTTT NATTTCCAAA AAAAAAAAAA AAAAA         2275
```

FIGURE 15

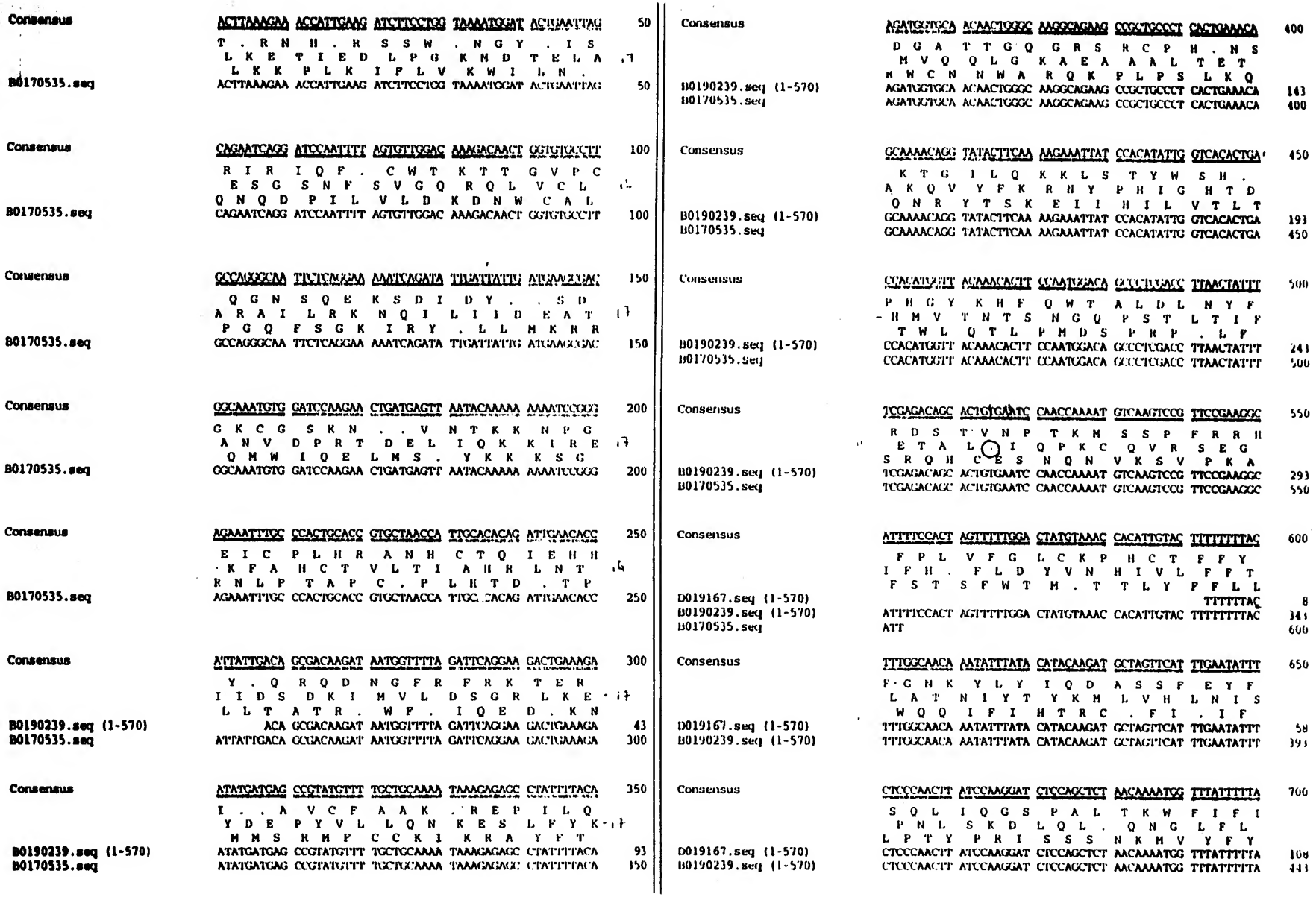


FIGURE 16

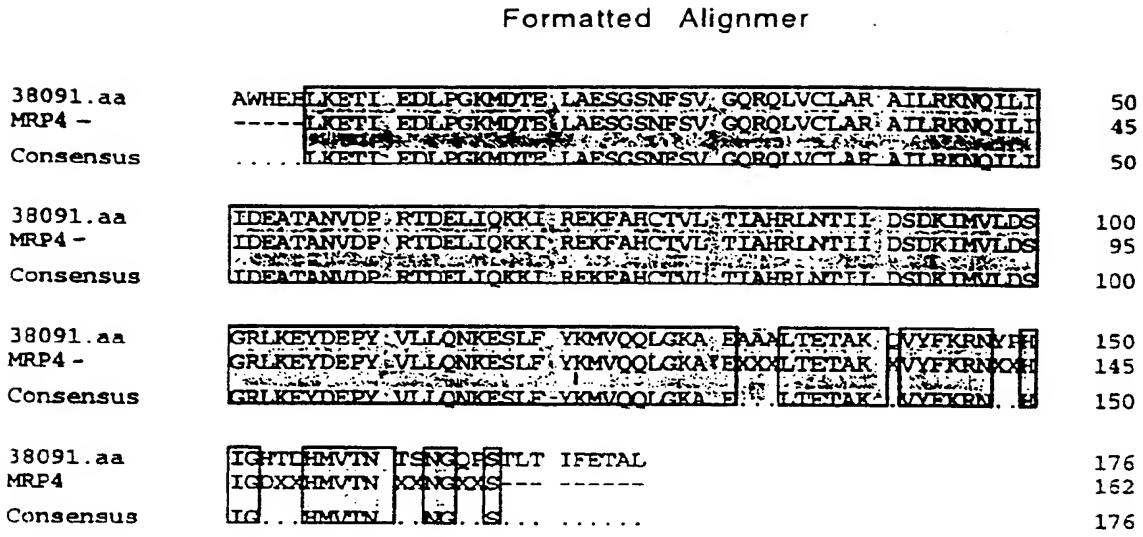

Formatted Alignmer
38091.aa
MRP4 -
Consensus
50
45
50
100
95
100
150
145
150
176
162
176

FIGURE 17

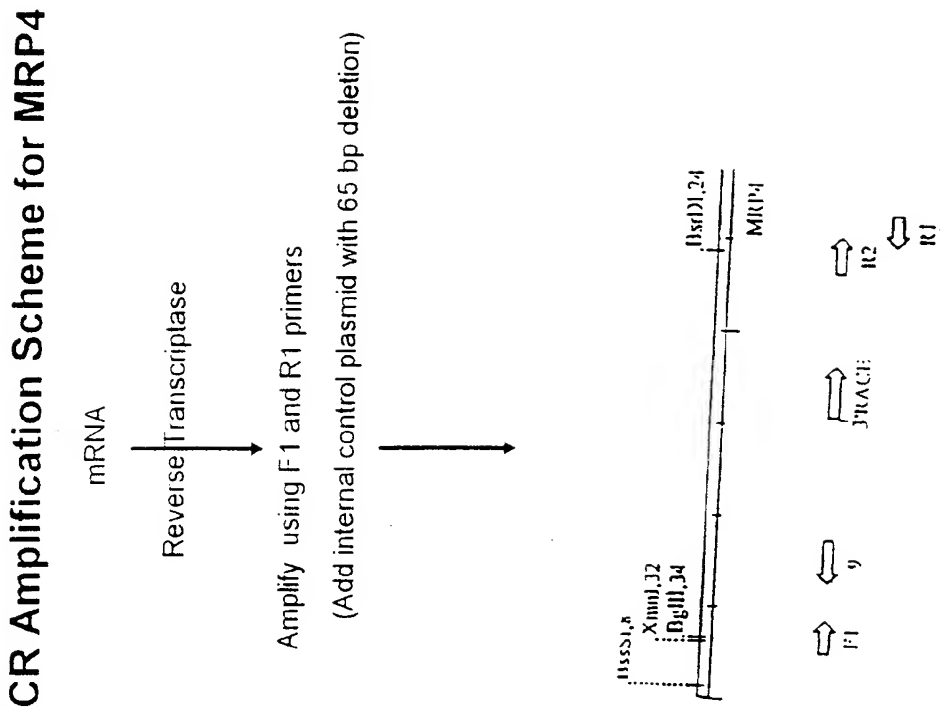

PCR Amplification Scheme for MRP4
mRNA
Reverse Transcriptase
Amplify using F1 and R1 primers
(Add internal control plasmid with 65 bp deletion)
MRP4
F1
R1
R2

FIGURE 18

FIGURE 19A MDR

```
                1                                                   50
crupgp1   VQILKGLNLK VQSGQTVALV GNSGCGKSTT VQLLQRLYDP TEGVVSIDGQ
crupgp2   IKILKGLNLK VQSGQTVALV GKSGCGKSTT VQLLQRLYDP TEGVVSIDGQ
humpgp1   VKILKGLNLK VQSGQTVALV GNSGCGKSTT VQLMQRLYDP TEGMVSVDGQ
pgpratlb  VKILKGLNLK VKSGQTVALV GNSGCGKSTT VQLLQRLYDP IEGEVSIDGQ
 muspgp   VQILKGLNLK VKSGQTVALV GNSGCGKSTT VQLMQRLYDP LEGVVSIDGQ
crupgp3   IKILKGLNLK VQSGQTVALV GNSGCGKTTT LQLLQRLYDP TEGTISIDGQ
humpgp3   VKILKGLNLK VQSGQTVALV GSSGCGKSTT VQLIQRLYDP DEGTINIDGQ
```

FIGURE 19B MRP

```
                                  .. . .. ... ....      . .    . . ...
          MRP1    FVLRHINVTI NGGEKVGIVG RTGAGKSSLT LGLFRINESA EGEIIIDGIN
          MRP2    LVLRGITCDI GSMEKIGVVG RTGAGKSSLT NCLFRILEAA GGQIIIDGVD
cMOAT/    MRP3    LVLRDLSLHV HGGEKVGIVG RTGAGKSSMT LACSRILEAA KGEIRIDGLN
          MRP4    .......... .......... .......... .......... ..........
          MRP5    ..........TI KPKEKIGIVG RTGSGKSSLG MALFRLVELS GGCIKIDGVR
          MRP6    LAVQGVSFKI HAGEKVGIVG RTGAGKSSLA SGLLRLQEAA EGGIKIDGVP
          HSUR    FVLKGTNALI SPGQKIGICG RTGSGKSSFS LAFFRMVDTF EGHIIIDGID
```

FIGURE 19C MRP4 PCR Product

FIGURE 19

5' Sequence of MRP4 2.7 kb PCR product Showing a Walker A Motif

GTGGGGCGGA CGGAGGCGGG GAAGTCATCA CTGGTAAGTG CCGTGCTCGG 50

NGAATTGGCC CCAAGTCACG GGCTGGTTAG CGTGCATGGA ANAATTGCCT 100

ATGTGTCTCA GCAGCCCTGG GTGTTCTCGG GAACTCTGAG GAGTAATATT 150

TTATTTGGGA AGAAATACGA AAAGGAACGA TATGAAAAAG TCATAAAGGC 200

TTGTGCTCTG AAAAAGGATT TACAGCTGTT GGAGGATGGN GATCTGACTG 250

TGATAGGAGA TCGGNGAACC ACGCTGAGTG GAGGGCNNAA AGCACGGGTA 300

AACCTTGCAA GAGCAGTGTA TCAAGATGCT GACATCTATC TCCTGGACGA 350

TCCTCTCANT GCAAGTANAT GCGGAAGTTA GCANACACTT GTTCGAACTG 400

TGTAATTTGN CANNTTTTGC ATGAGAAGAT CACAATTTTA ATCGACTCAT 450

NAAGTNTGCA AGTACCTCAA AGCTGCAAGT CNNATTCTTG ATANTGAAAG 500

ATGGTAAAAT GGNGCCAAAA NGGNACTTAC ACTTGAGCTT CCTAAAATCT 550

GGNATACAAT TTNGCTTCCT TTTAAANAAA CGATAA 586

FIGURE 20

Query= M11 For Sequence of 2.7kb MRP4 PCR prod (5' end seq (586 letters)

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences 344 964 sequences; 722,615 370 total letters.

Searching..... done

Sequences producing High-scoring Segment Pairs		Smallest Sum Probabil P(N)
emb|X96393|RNCMRP	R.norvegicus mRNA for canalicular mu...	539
gb|L49379|RATCMOAT	Rattus norvegicus canalicular multis...	539
dbj|D86086|D86086	Rat mRNA for canalicular multispecif...	539
gb|U66261|CEU66261	Caenorhabditis elegans multidrug res...	469
gb|AC002411|F20D22	Arabidopsis thaliana chromosome 1 BA...	468
emb|Y11250|ATMRPPROT	Arabidopsis thaliana mRNA for putati...	465
gb|U41015|CELF20B6	Caenorhabditis elegans cosmid F20B6	457
gb|U63970|HSU63970	Human canalicular multispecific orga...	451
emb|X96395|HSCMRP	H.sapiens mRNA for canalicular multi...	451
gb|U49248|HSU49248	Human canalicular multispecific orga...	451
emb|Z48179|SC9302X	S.cerevisiae chromosome IV cosmid 9302	426
gb|L35237|YSCYCP1MRP	Saccharomyces cerevisiae metal resis...	426
gb|U92650|ATU92650	Arabidopsis thaliana MRP-like ABC tr...	422
gb|U41554|CELF57C12	Caenorhabditis elegans cosmid F57C12.	284
emb|X65256|XLCFTRM	X.laevis CFTR mRNA	381
gb|U20418|OAU20418	Ovis aries cystic fibrosis transmemb...	358
gb|M76128|BOVTCRCF	B.taurus cystic fibrosis transmembra...	349
gb|S82688|S82688	CFTR=cystic fibrosis transmembrane c...	339
gb|AF013753|AF013753	Macaca mulatta cystic fibrosis trans...	349
gb|M28668|HUMCFTRM	Human cystic fibrosis mRNA, encoding...	340
gb|U60209|XLU60209	Xenopus laevis cystic fibrosis trans...	345
gb|M60493|MUSMCFTR	Mouse cystic fibrosis transmembrane ...	321
gb|M84613|MUSCFTRY	Mouse cystic fibrosis transmembrane ...	321
gb|U40227|OCU40227	Oryctolagus cuniculus CFTR chloride ...	322
gb|M69298|MUSCFTR	Mouse cystic fibrosis transmembrane ...	322
gb|U33010|SPU33010	Schizosaccharomyces pombe cosmids 35...	303
gb|AF022908|AF022908	Mus musculus multidrug resistance pr...	294
gb|M89996|RATCFTR	Rattus norvegicus cystic fibrosis tr...	285

emb|X96393|RNCMRP R.norvegicus mRNA for canalicular multidrug resistance protein
Length = 4909

Plus Strand HSPs:

Score = 539 (148.9 bits), Expect = 1.8e-34, P = 1.8e-34
Identities = 209/340 (61%), Positives = 209/340 (61%), Strand = Plus / Plus

```
Query:    17 CGGGGAAGTCATCACTGGTAAGTGCCGTGCTCGGNGAATTGGCCCCAAGTCACGGGCTGG 76
             |||||  ||  ||  |||||   ||| |||| ||  ||| |||          |||||||||
Sbjct:  2061 CTGGGAAATCCTCTTTGGTATCAGCCATGCTGGGAGAAATGGAAAACGTTCACGGGCACA 21

Query:    77 TTAGCGTGCATGGAANAATTGCCTATGTGTCTCAGCAGCCCTGGGTGTTCTCGGGAACTC 13
             | | | ||  |||   | ||||||||  ||||||||  |||||  |      |||||
Sbjct:  2121 TCACCATCCAGGGATCCACAGCCTATGTCCCTCAGCAGTCCTGGATTCAGAATGGAACCA 21

Query:   137 TGAGGAGTAATATTTTATTTGGGAAGAAATACGAAAAGGAACGATATGAAAAAGTCATAA 19
             | |   ||  ||  | ||||||    |||||  |  |  |   ||  |  ||||  | |
Sbjct:  2181 TCAAAGACAACATCCTGTTTGGGTCCGAATACAATGAAAAGAAGTACCAGCAAGTTCTCA 22

Query:   197 AGGCTTGTGCTCTTGAAAAAGGATTTACAGCTGTTCGAGGATGGNGATCTGACTGTGATAG 25
             | ||  ||  |||||          ||  ||  |  |  |||   |  ||  ||  ||| |||
Sbjct:  2241 AAGCATGCGCTCTTCTCCAGACTTGGAAATATTGCCTGGAGGAGACATGGCTGAGATCG 23

Query:   257 GAGATCGGNGAAGCAGCTGAGTGGAGGGCNAAAGCACGGGTAAACCTTGCAAGAGCAG 31
             ||||   |  |  |  ||  |||||  ||  |  ||  ||  |  ||  ||  |||||  |
Sbjct:  2301 GAGAGAAGGGGATAAATCTCAGTGGTGGTCAGAAGCAGCGAGTCAGCCTGGCCAGAGCTG 23

Query:   317 TGTATCAAGATGCTGACATCTATCTCCTGGACGATCCTCT 356
             |||||||||||||||||||||  |||||||||||  ||
Sbjct:  2361 CCTATCAAGATGCTGACATCTATATTCTGGACGATCCCCT 2400
```

FIGURE 21

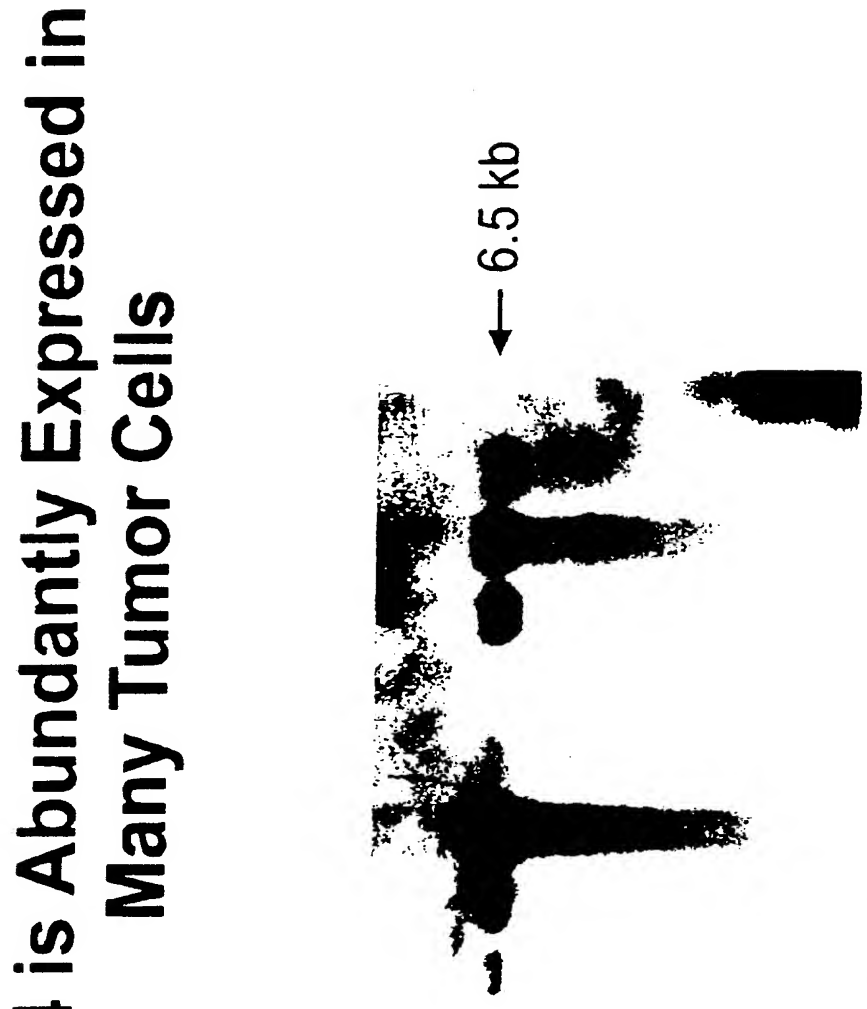

MRP4 is Abundantly Expressed in Many Tumor Cells
6.5 kb
HL60
Hela
K562
Molt4
RAJI
SW480
Lung A459
Melanoma

FIGURE 22

MRP4 Expression is Down-regulated by p53 in K562 Erytholeukemia Cells

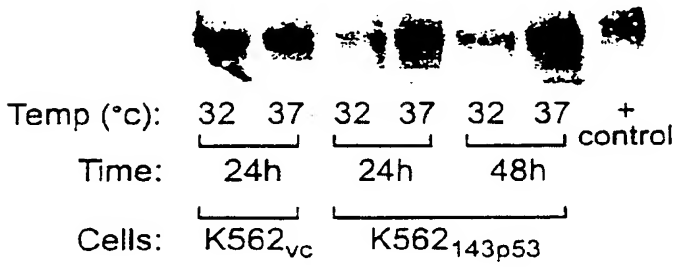


FIGURE 23

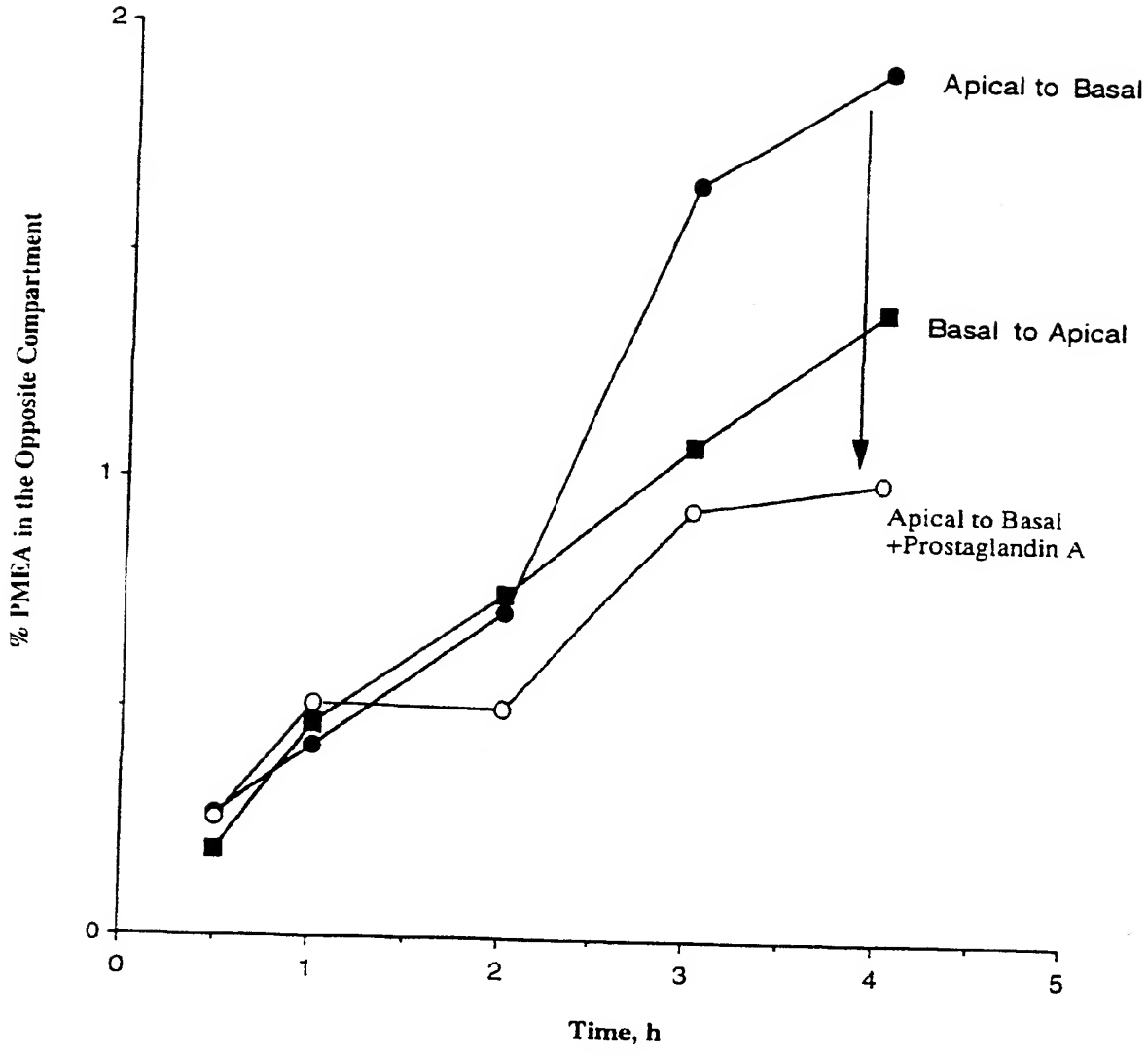

2
1
0
0
1
2
3
4
5
% PMEA in the Opposite Compartment
Time, h
Apical to Basal
Basal to Apical
Apical to Basal
+Prostaglandin A

FIGURE 24

PMEA Resistant CEM Cell Lines
Have Increased MRP4
Gene Copy and Expression

FIGURE 25A

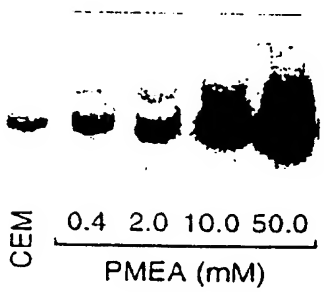


FIGURE 25B

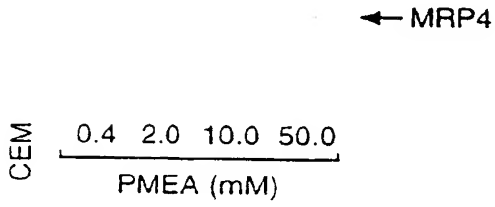


FIGURE 25C

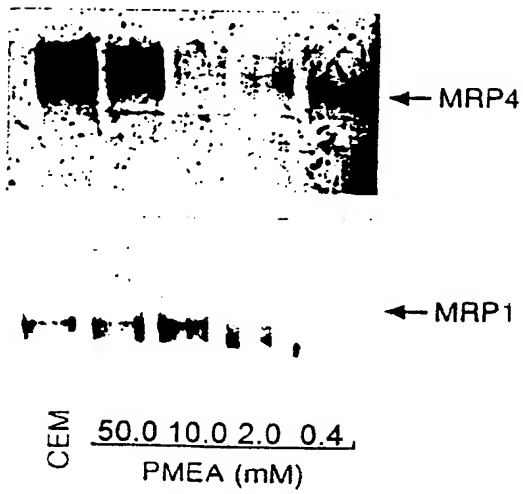


FIGURE 25

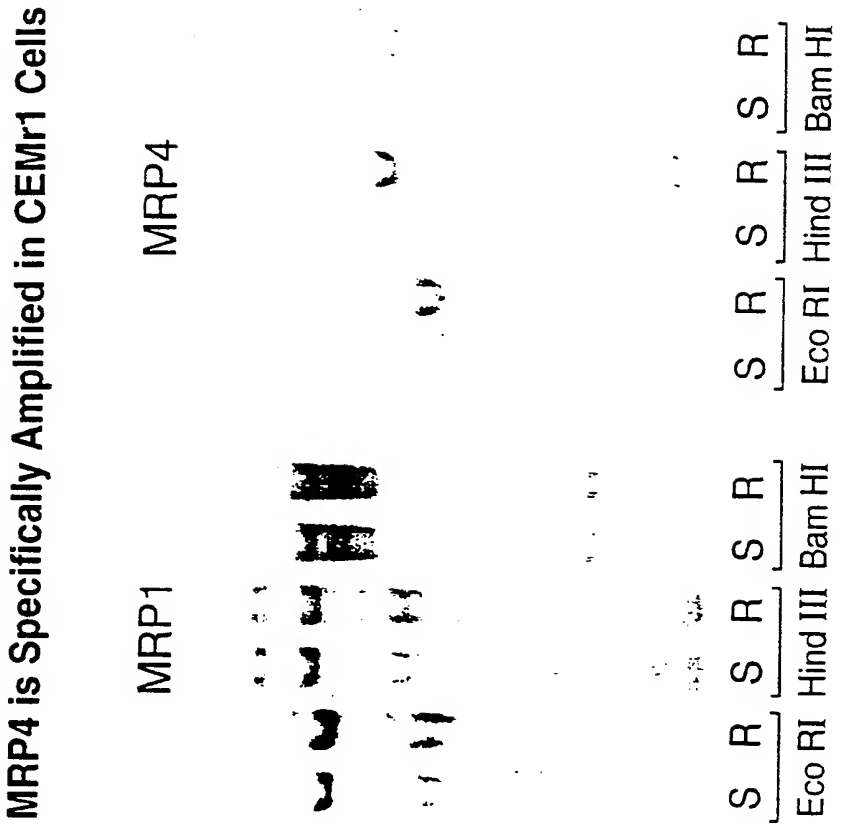

MRP4 is Specifically Amplified in CEMr1 Cells
MRP1
MRP4
S R S R S R
Eco RI Hind III Bam HI
S R S R S R
Eco RI Hind III Bam HI

Figure 26

MRP4 Antibody Reveals MRP4 is Selectively Increased in PMEA Resistant CEM Cells

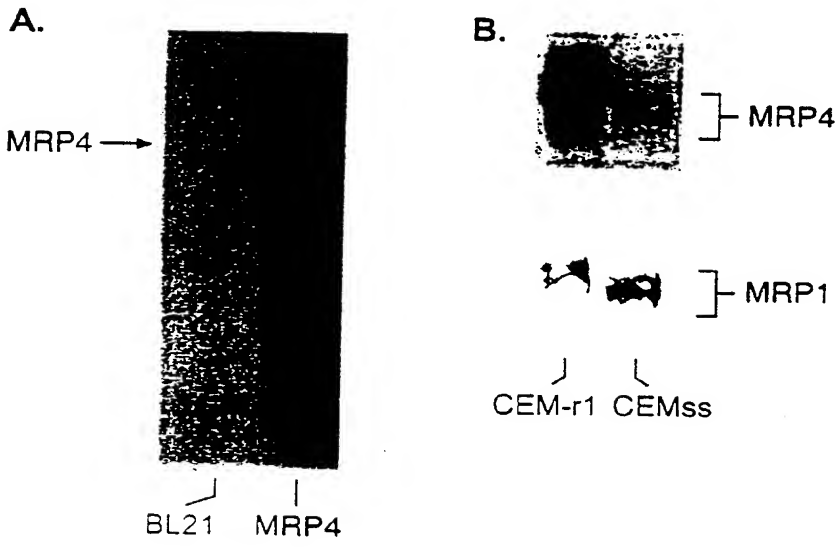


Figure 27

PMEA Resistant CEM Cell Lines Have Increased MRP4 Gene Copy and Expression

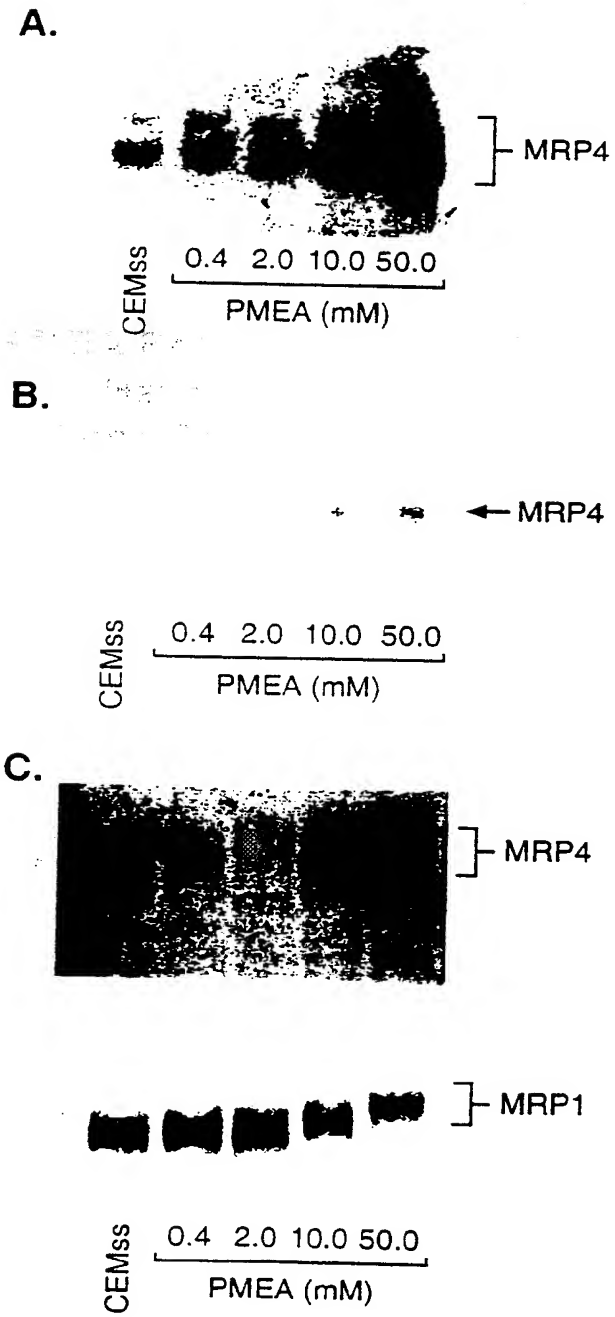


Figure 28